Microbial associations of Greek meat with special emphasis on fermented sausages

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MICROBIAL ASSOCIATIONS OF GREEK MEAT WITH SPECIAL EMPHASIS ON FERMENTED SAUSAGES

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Contents

TITLE	PAGE
AKNOWLEDGEMENTS	
SUMMARY	1
INTRODUCTION	3
CHAPTER 1	
Literature Review	5
CHAPTER 2	
Materials and Methods	36
Section A; Meat stored under different gaseous conditions	36
Section B; Staphylococci of Greek Salami	41
Section C; Laboratory studies of sausage fermentation	44
Section D; Studies of the factors that affect the growth and physi chemical attributes of *Lactobacillus plantarum and Staphylococcus carnosus* in broths	46
CHAPTER 3	
Meat stored under different gaseous conditions	49
Introduction	49
Materials and Methods	51
Results	52
Discussion	72
CHAPTER 4	
The staphylococci of Greek salami	78
Introduction	78
Materials and methods	79
Results	79

CHAPTER 5
Laboratory studies of sausage fermentation 91
Introduction 91
Materials and Methods 93
Results 93
Discussion 147
CHAPTER 6
Studies of the factors that affect the growth and physicochemical attributes of *Lactobacillus plantarum* and *Staphylococcus carnosus* in broths 150
Introduction 150
Materials and Methods 152
Results 152
Discussion 184
GENERAL DISCUSSION 188
APPENDIX 191
REFFERENCES 193

Summary

The inhibitory effect of CO_2 on Gram-negative aerobic psychrotrophic meat bacteria was exemplified by the results of this study. A shift from an initial flora containing Gram-negative aerobic organisms to a Gram-positive facultative anaerobic microflora dominated by *Lactobacillus* spp. occurred under Modified Atmosphere Packaging (MAP) storage. The shelf-life of minced beef or pork (normal and high pH) stored in CO_2 at 3° C, was almost doubled.

Little attention has been given to the occurrence of staphylococci and their activity in salami-dry sausages in Greece. A survey was done in order to characterise some physicochemical and microbiological attributes of salami (dry sausage) produced by the 7 companies that command the market for this product in the metropolitan area of Athens. *Staphylococcus carnosus* was isolated and identified in several samples. Of 100 isolates 17 were identified as *St. carnosus*. The "back-slop" technique used traditionally in Greece to inoculate sausage seems to favour the selection of this species.

The best simulation of sausage production in the laboratory was obtained with sausage meat inoculated with *Lactobacillus plantarum* and *Staphylococcus carnosus* in combination, stuffed into natural casings and incubated in moving air. The pseudomonads and Enterobacteriaceae counts changed to a limited extent during fermentation. On the other hand the lactic acid bacteria with or

without addition of starter cultures multiplied vigorously and became dominant in the first few days of fermentation. The micrococaceae increased and became the second most dominant organisms. L-lactic acid, produced as a metabolic product, was estimated. Gas-liquid chromatography showed that acetic acid and ethanol were produced also. This was taken as evidence of heterolactic fermentation.

The effect of the composition of different growth media (broths) with or without glucose and oxygen limitation on the accumulation of metabolic products such as lactate and acetate and on the formation of exoproteins which may contribute to the characteristic attributes of the final product was investigated in another part of the study. The results of this part of the study indicated that in the absence of a readily available source of energy, *St. carnosus* and *Lactobacillus plantarum* cultures grown in media appeared to make use of *iso*- Lactate Dehydrogenase (iLDH) by using lactate as a carbon source oxidising further to acetic acid or to stimulate the TCA cycle. Indeed, lactate was produced initially in the various media under static or shaking conditions and it was utilised in the latter stages of incubation in all samples inoculated with these two organisms in combination or alone.

Introduction

The Greek meat market is very traditional. Fresh meat still tends to be bought on the day it is to be used and small butchers rather than supermarkets account for the bulk of the sales. As yet there is no evidence of fresh meat in modified atmosphere packs being widely available in supermarkets. A section of this thesis deals with minced meat stored in modified atmospheres. This phase of the study had two objectives. Firstly to monitor changes in the populations of particular groups of microorganisms and to identify broadly those responsible for spoilage. Secondly to study physicochemical changes in meat such that information is available should future investigators in Greece seek chemical rather than microbiological methods for the assessment of the freshness of meat. As far as can be acertained, the present study is the first of its kind to be done in Greece.

A wide variety of dry, semi-dry and other forms of sausages has been an important part of the Greek diet over many centuries. The range of sausage types can be ascribed to the geography of Greece, a relatively small country with a large number of islands. It led to the evolution of particular types of sausages in regions and, more especially, on particular islands. Today the most popular sausages tend to be made by relatively large manufacturers for regional or even national distribution. Even though the scale of operation has increased, initiation of fermentation in sausages had to be based until recently on a traditional system, back-slopping, because Greek law forbade the use of starter cultures. The law was changed in 1988 and starter cultures can now be used by manufacturers.

Although there is a very long history of production of fermented sausages in Greece,there are only 4 publications (Paneras and Bloukas 1984, Bloukas and Paneras 1985, Grigoriadis et al. 1985,1988) dealing with the technological aspects of this commodity. In practice, none of these dealt in any detail with the microorganisms responsible for fermentation of the major products. This thesis presents information on three aspects of fermented Greek sausages.

1. The occurrence of *Staphylococcus carnosus* in sausages made by large manufacturers whose products dominate the markets in the metropolitan area of Athens.
2 The behaviour of *Lactobacillus plantarum* and *Staphylococcus carnosus* either in pure or mixed cultures in laboratory media.
3. The contribution of these two species to sausage fermentation in laboratory studies.

Chapter 1

Literature Review

Microbiology: Numbers and types of micro-organisms in meat

1. Raw Meat

Contamination and Spoilage

As the inherent antimicrobial defence mechanisms of the live animal are destroyed at slaughter, the resultant meat is liable to rapid microbial decay. Unless effectively controlled, the slaughtering process may cause extensive contamination of the cut face of muscle tissue with a vast range of both Gram-negative and Gram-positive bacteria as well as yeasts (Table 1.1). Some of these microorganisms will be derived from the intestinal tract and others from the environment with which the animal had contact at some time before slaughter. Today the spoilage of meat in developed countries is caused by the selection of relatively few of these organisms (Table 1.2). It is evident from Table 1.3 that chill storage and the gaseous composition around meat packed in vacuum or in modified atmospheres exerts very strong selective pressures. Only a few studies have been done with yeasts on meat (Table 1.1). None of these has demonstrated yeast involvement in meat spoilage (Dillon and Board 1991). It is well known (Mossel and Ingram 1955) that the spoilage of a food under particular conditions of storage is caused by only some of the microbial contaminants present initially. In practice selective factors favour the growth of particular organisms and, as a consequence, a characteristic microbial association is present at the time of spoilage and it will manifest characteristic spoilage features. With the advent of supermarkets in the late 1950's, for example, chill storage became a major selective factor in meat spoilage and *Pseudomonas* spp.

Table 1.1 Genera of organisms recovered from red meat and products not manifesting signs of incipient spoilage[+]

Bacteria		
Gram-negative		
Aerobes	**Facultative anaerobes**	
Pseudomonas	Escherichia	
Acinetobacter	*Pantoea*	
Achromobacter	*Serratia*	
Flavobacterium	*Hafnia*	
Alcaligenes	*Citrobacter*	
Moraxella	*Vibrio*	
Psychrobacter		
Gram-positive		
Aerobes	**Facultative anaerobes**	**Obligate anaerobes**
Bacillus	*Bacillus*	
Micrococcus	*Staphylococcus**	*Clostridium*
Kurthia	*Brochothrix*	
	Lactobacillus	
	Carnobacterium	
	Enterococcus	
	Lactococcus	
	Leuconostoc	
Yeasts		
Micro-fungi**		

[+] Based on Mossel and Ingram (1955), Ayres (1960a) and Dainty and Mackey (1992) and, with yeasts, Dillon and Board (1989)

* The occurrence of this genus on meat and meat products is summarised in Table 5 of the review reproduced from the Journal Applied Bacteriology (p. 25)

** Microfungi. A range of these organisms have been isolated from meat and meat products over the years but only some, ie *Cladosporium herbarum*, have been of commercial immportance. This species discoloured the surface of carcasses during refrigerated shipment from the antipodes to the UK before the second World War

Table 1.2 Psychrophilic[a] and psychrotrophic bacteria associated with chilled meats and meat products *

Family/species	Comments References
Gram-negative bacteria-Aerobes	
Moraxelaceae[b]	
Psychrobacter immobilis	Oxidase-positive members of this family; some strains form acid (aerobically) glucose; some strains probably identified with Achromobacter in early studies. Bovre (1984); Juni and Heym (1986); Rosseau et al ,(1987).
Acinetobacter	Oxidase-negative members of this family; many strains show marked nutritional versatility; many contain an aldose dehydrogenase and form gluconic acid; some strains probably identified with Achromobacter[c] in early studies 12 hybridzation groups (genospecies) among 85 Acinetobacter strains included in a recent study (Bouvet and Grimont 1986).
Pseudomonaceae	
Pseudomonad[d] rRNA homology Group 1 Pseudomonas fluorescens Biovars I,II,III , IV,V (includes 7 clusters) Ps. lundensis Ps. fragi	Oxidase-positive,glucose-oxidizing organisms with marked nutritional versatility. The order of species is the inverse of the incidence of individual species on meat. Shaw and Latty (1982); Banks and Board (1983); Molin and Ternstrom (1986); Palleroni et al. (1973); Palleroni (1984); Molin and Ternstrom (1982); Barrett et al. (1986); Molin et al. (1986).
Gram-negative bacteria - Facultative anaerobes	
Vibrionaceae	More commonly a spoilage organism on
Shewanella putrefaciens *	fish and poultry than on red meat. Vacuum packaging of low-acid (pH>6.0) red meats is associated with growth of this organism; H_2S production from amino acids causes greening. Gill (1986); MacDonell and Colwell (1985).
Enterobacteriaceae[f]	Many members of this family have been isolated from meat works and meat,viz, Enterobacter, Serratia, Hafnia, Citrobacter. Some species, eg. Pantoea liquefaciens grow on vacuum-packed meat of pH 6.05. Gill and Newton (1982).

Table 1.2 (continued)

Gram-positive bacteria-	
Facultative anaerobes	
Catalase reaction-weak	
Brochothrix thermosphacta[a]	L-(+)-Lactic acid formed from anaerobic and acetoin, isobutyric and isovaleric acids from aerobic utilization of glucose. Enzymes of TCA cycle are almost totally absent. Growth factors required. Sneath and Jones (1976).
Catalase reaction-negative	
Lactobacillus [b]	An inadequately studied group of acid-tolerant bacteria notable for absence of TCA cycle-hence, dependent upon exogenous sources of amino acids-and requirement for growth factors and a fermentation substrate, viz, glucose, from which lactic acid (homo-fermentative) or CO_2, lactic and acetic acids (heterofermentative) are formed. Shaw and Harding (1984,1985); Hastings and Holzapfell (1987); Beuter (1981); Egan (1983); Collins et al. (1987); Hiu et al. (1984); Holzapfell and Serber (1983).
Carnobacterium	
Leuconostoc	
Lactococcus	
Psychrophilic	
Clostridium sp.	An as yet unidentified species of this has been associated with the distension and, in some cases, explosion, of vacuum packed meat stored at chill temperatures. It has been shown to match the criteria of a true psychrophile. Dainty and Mackey (1992).

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[a]. For a review of this definition of psychrotroph and psychrophile and the biochemical attributes of these two groups of bacteria, see Gounot (1986).

[b]. A study of inter- and intragenetic similarities of ribosomal ribonucleic acid cistrons indicated that this family contains at least five unrelated groups (Rossau et al.1986). One group containing Acinetobacter, Moraxella, and misnamed Achromobacters is somewhat related to organisms belonging to rRNA superfamily II which contains the Pseudomonas fluorescens complex. See Rosseau et al.(1987) for further discussions of rRNA cistron similarities among members of this family.

Table 1.2 (continued)

[c]. Hendrie et al. (1984) proposed that Achromobacter be considered as a nomen dubiem. Recently the genous has been reinstated to accomodate isolates from clinical sources (Yabunchi and Yand 1981).

[d]. The pioneering study based on phenotypic properties (Stanier et al. 1966) in the modern era of Pseudomonas taxonomy did not include the organisms Ps. fragi and Ps. lundensis, of common occurrence on chilled meat, a feature first noted by Davidson et al. (1973). Subsequent studies based on an analysis of 16s RNA have confirm the close relationship of these three species (Willems et al. 1992).

[e]. This organism has suffered from numerous changes in name, viz, Pseudomonas putrefaciens (Long and Hammer 1941), Achromobacter putrefaciens (Derby and Hammer 1931), and Alteromonas putrefasciens (Lee et al. 1981). Van Landschoot and De Ley (1983) concluded from a study of the rRNA cistrons that the genus Alteromonas was very like those of Vibrionaceae. Their findings were reflected in the study by McDonnell and Colwell (1985) who proposed that this species be renamed Shewanella putrefaciens and who recommended a revision of the definition of Vibrionaceae.

[f]. Pantoea agglomerans, a species occuring in unsulfited British-style sausage (Banks and Board 1982), has been the subject of a recent taxonomic studies (Verdonek et al. 1987 ; Gavini et al. 1983,1989).

[g]. This organism, which was originally isolated from American pork sausages (Sulzbacher and MacLean 1951), was named Microbacterium thermosphactum by McLean and Sulzbacher (1953). The currently accepted name was proposed by Sneath and Jones (1976). It is an important spoilage organism of vacuum-packed sliced meat products (Qvist and Hukherji 1981). Confusion of this organism with lactobacilli and streptococci is possible if incorrect procedures for the catalase test are used (Davidson et al. 1968). It must be stressed that Br. thermosphacta exhibits a growth cycle (long rods->short rods->coccobacilli) during growth in the laboratory (Davidson et al. 1968). A similar growth cycle is found in two occasional contaminants of meat, Kurthia zopfii and K. gibsonii (Shaw and Keddie 1983). Motility and a strong catalase reaction distiguish the latter from the former.

[h]. Members of this genus tend to be associated with radurized meat (Hastings and Holzapfell 1987).

Table 1.3 The numerically dominant organisms of the microbial associations that develop on jointed or minced red meats under different storage conditions

Storage condition	Organisms selected	References
Normal atmosphere at 2-7 °C	*Pseudomonas fluorescens*	1
	fragi	1
	lundensis	1
	Psychrobacter immobilis	1,2,3,4,5
	Acinetobacter johnsonii	1,2,3,4,5
Modified atmosphere with CO_2 added or vacuum packs at 2-7° C	*Brochothrix thermosphacta*	1,2,3,4,5
	Enterobacter liquefaciens	1,2,3,4,5
	Lactobacilus sake	1,2,3,4,5
	curvatus	
	bavaricus	
	Carnobacterium divergens	1,2,3,4,5
	piscicola	
	Leuconostoc carnosum	1,2,3,4,5
	gelidum	
	mesenteroides subsp. *mesenteroides*	
	Lactococcus raffinolyticus	1,2,3,4,5
	Clostridium sp.	1,2,3,4,5

1 : Molin and Ternstrom (1982)
2 : Shaw and Latty (1982)
3 : Shaw and Latty (1984)
4 : Molin and Ternstrom (1986)
5 : Dainty and Mackey (1992)

were considered to be the main spoilage organisms of meat held under aerobic, chilled conditions which did not cause the surface of the meat to dry out (Wolin *et al.* 1957; Ayres 1960b; Gardner 1965; Stringer *et al.* 1969). As there tends to be an inverse relationship between the initial numbers of spoilage organisms on meat and the time required for spoilage to occur (Sumner 1978; Nottingham 1982), the cleanliness of equipment used in butchering and mincing of meat as well as the temperatures obtaining not only during preparation but in subsequent storage will all play a role in determining the rate of microorganisms selection and shelf life (Rogers and McCleskey 1957). Thus, for instance, products prepared in a controlled and specifically designed processing operations for minced meat were of a better microbiological quality than those produced by traditional methods in butchers' shops because of improved standards of hygiene (Shoup and Oblinger 1976).

Other studies have been concerned with the origin of the contaminants noted in Table 1.2. In a study of two packaging plants (Stiles and Ng 1981) the source of Enterobacteriaceae on meats, for example, was shown to be associated with the work surfaces. Newton *et al.* (1978) recovered psychrotrophic bacteria from hides and work surfaces within an abattoir as well as from carcasses and butchered meat at all stages of processing.

Currently in the UK a consumer may buy meat from a traditional butcher's shops or from supermarkets. In the latter case the meat may or may not be offered in a modified atmosphere pack. Nychas *et al.* (1991a) surveyed minced beef from both sources and, with the supermarkets, minced meat packaged for the consumer's convenience or in modified atmospheres. They used various selective media to characterize the microbial associations and a range of physicochemical techniques to follow changes in meat during chill storage. The principal observations of this study are summ arised in Table 1.4. It is evident from this Table that pseudomonads became dominant in butchers'

Table 1.4 Data obtained from the survey of minced beef during July-August (J-A) and November-December (N-D)*

	Type of packaging					
	1	2	3	1	2	3
	J - A			N - B		
Total Viable Counts	7.39[a]	8.85	8.89	6.77	8.10	8.65
Pseudomonads	nd	nd	nd	5.78	7.57	8.57
Enterobacteriaceae	4.65	5.96	6.07	3.70	5.21	5.54
Lactobacilli	5.33	6.70	6.31	4.60	5.45	5.00
Br. thermosphacta	5.72	7.30	8.30	5.10	6.50	7.70
Yeasts	4.60	5.10	6.10	3.80	4.75	5.40
pH	5.75	5.80	5.99	5.75	5.90	6.05
TA[b]	2.49	2.80	3.84	2.15	2.55	2.90
ERV[c]	43.3	37.9	36.8	63.8	58.9	53.5
Total Sugars[d]	359	254	195	360	285	227
Glucose[d]	nd	nd	nd	88.9	76.1	66.2

1 : flushed with CO_2/O_2
2 : wrapped with flexible transparent film
3 : minced beef in a plastic bag atthe time of purchase
a : $\log_{10}$ cfu g^{-1} minced beef
b : titrimetric acidity; ml of 0.02 M HCL
c : extract release volume (ml)
d : mg of substance 100 g^{-1} minced beef
nd : not-determined

* Adapted from Nychas *et al.* (1991a)

mince during storage at 4° C whereas Gram-positive bacteria did so in mince in a modified atmosphere (20% O_2; 80% CO_2). The results presented in Table 1.4 show also that these different microbial associations were associated with marked differences in the Extract Release Volume and the pH of stored minced. In addition it was demonstrated that changes in the concentration of gluconate was a feature only of minced meat stored in air. It was deduced that the changes in gluconate concentration were due to the metabolism of *Pseudomonas* spp. This topic has been reviewed by Nychas *et al.* (1988).

Microorganisms of the spoilage association

Over the years the majority of the Gram-negative aerobic psychrotrophic bacteria of meat were identified with a number of ill defined species of *Pseudomonas* - *Ps. fluorescens*, *Ps. fragi*, *Ps. putida*, *Ps. geniculata* etc. (Kirsch *et al.* 1952, Wolin *et al.* 1957, Ayres 1960a,b; Gardner 1965, Davidson *et al.* 1973, Nychas 1984, Molin and Tenstrom 1982, Banks and Board 1983). The pioneering study of Stanier *et al.* (1966) provided phenotypic properties that improved the definition of species worthy of inclusion in this genus. Subsequent studies based on the analysis of 16s RNA have also improved the definition of species (Willems *et al.* 1992). Of the many species included in the genus *Pseudomonas*, three are of particular importance in meat microbiology, *Ps. fragi*, *Ps. fluorescens* and *Ps. lundensis*.

Although rarely, if ever, contributing significantly to the spoilage flora on meat and meat products, Enterobacteriaceae have been considered as indicators of food safety by Mossel (1962) and Mousa *et al.* (1973). Stiles and Ng (1981), who studied 2343 isolates of Enterobacteriaceae, concluded that these organisms were introduced onto meat from work surfaces and not by direct feacal contamination. With ground beef, *Pantoea*

agglomerans, *Esch. coli*, and *Ser. liquefaciens* were the major representatives of this family (Nychas *et al.* 1988). *Brochothrix thermosphacta* (Sneath and Jones 1976) - formerly known as *Microbacterium thermosphactum* (McLean and Sulbacher 1953) - has been classified within the Lactobacillaceae. *Brochothrix thermosphacta* has been detected in the aerobic spoilage flora of chilled meat (Gardner *et al.* 1967) but it is not considered to be important in spoilage except possibly of lamb (Barlow and Kitchell 1966). This organism has been isolated from beef carcasses during boning, dressing and chilling (Newton *et al.* 1978). Moreover lairage slurry, cattle hair, rumen contents, soil from the walls of slaughter houses, the hands of workers, air in the chill room, neck and skin of the animal as well as the cut muscle surfaces have been shown to be contaminated with this organism (Mulder 1978; Patterson and Gibbs 1978). According to Gardner (1981) it grows in certain comminuted, uncured meats, for example fresh pork sausage. *Brochothrix thermosphacta* is one of the main, if not the most important, cause of spoilage which can be recognised as souring rather than putrefaction. This form of spoilage is commonly associated with meat packed in modified atmospheres.

At one time members of the Lactobacillaceae were not considered to be spoilage organisms of meat. With the advent of vacuum or modified atmosphere packaging, these organisms were recognised as important members of the spoilage association (Table 1.3). Many of the isolates could not be identified with existing species of *Lactobacillus* (Table 1.3). It is now recognised that many of these isolates are species of a recently defined genus, *Carnobacterium* (Dainty and MacKey 1992).

To date the contribution of yeast to the spoilage of meat, whole or minced, has attracted little attention even though they are common contaminants (Hurst 1972, Jay and Margitic 1981, Dalton *et al.* 1984 , Dillon *et al.* 1991). According to Dillon and Board (1991) yeasts do not

outgrow bacteria on meat or meat products unless a bacteriostatic agent is included, such as sulphite in British fresh sausages, or the water activity is reduced. As noted above, the advent of supermarkets introduced effective chill storage for meat and meat products offered to the housewife. The selective pressures favoured the growth of cold tolerant microorganisms. Initially there was confusion in the terms used to describe such organisms. Some authors used the term psychrophile while other used psychrotroph (Mossel and Ingram 1955). The recent review by Gounot (1986) ought to lead to the appropriate choice of term in future studies. Her definition of psychrotroph means that, with but one exception, the bacteria associated with meat spoilage are of this physiological type. It is only within the past 3 years or so that a worthy candidate for the term psychrophile has been identified. Two studies to date (Dainty and Mackey 1992) have isolated an as yet unidentified *Clostridium* sp. that causes distension or explosion of packs of vacuum packaged meat. The optimum growth temperature of these organisms is 20° C. It is tempting to speculate that the production of a spore protects this organisms from those factors in meat processing that kills psychrophiles lacking this means of protection.

The above review is based on work done in Northern Europe, North America and New Zealand, areas of temperate climates. There is evidence that meat produced in warmer climates may harbour few psychrotrophic bacteria (Mossel and Ingram 1955) and hence have a slightly longer shelf life at chill temperatures. As Greece has a climate different from those in which the majority of studies of meat microbiology has been done, one part (Chapter 3) of the present study considered the microbiology of minced meat of Greek origin in this context.

2. Chemistry of spoilage

It is well documented by several workers that

microbial growth and thereby spoilage of meat and meat products occurs at the expense of low molecular weight soluble components, while the macromolecules are unaffected (Gill and Newton 1978). On the other hand the concentrations of the low molecular mass compounds are all sufficient to support massive microbial numbers (Gill 1976; Newton and Gill 1978a,b,c). The order in which these substrates are attacked by the various groups of spoilage bacteria is presented in Table 1.5. The relative potential of bacteria depends upon which species predominate, and upon their ability to form malodorous compounds, such as H_2S, volatile amines, esters and acetoin (McMeekin 1982; Lambert *et al.* 1991). Glucose is utilized by almost all the species of bacteria which are known to grow on meat stored with or without O_2. Additionally, glucose-6-phosphate, amino acids and lactic acid are utilized by some of these organisms as well (Gill 1976; Newton and Gill 1978a,b,c; Dainty and MacKey 1992). Potentially catabolites of all of the various types of bacteria growing under any particular storage conditions could contribute to the chemical changes perceived as off-odours, off-flavours or discolorations.

Pseudomonad species growing on the surface of meat will preferentially consume glucose until the rate of diffusion of glucose from underlying tissues becomes inadequate to meet their demand (Gill 1976). When high numbers (10^8 cm^{-1}) are reached and glucose becomes depleted at the meat surface, pseudomonads start to use amino acids as their growth substrate with production of malodorous sulfides, esters and acids (Gill 1976, McMeekin 1982).

Acinetobacter\Moraxella constitute a major part of the aerobic spoilage population. These organisms are of low spoilage potential. They utilize amino acids as their growth substrate but do not form malodorous by-products from amino acid degradation (Gill and Newton 1977). They rather enhance the spoilage activities of pseudomonads and *A. putrefaciens* by restricting the availability of O_2

(**Table 1.5**. Substrates used for growth and metabolic by-products of major meat spoilage microorganisms[a].

Microorganism	Substrates used for growth[b]		Major end products of metabolism	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Pseudomonas	Glucose[1] Amino acids[2] Lactic acid	-	Slime Sulfides Esters, acids, amines	-
Acinetobacter/ Moraxella	Amino acids[1] Lactic acid[2]	-	Esters, nitrites, oximes, sulfides	-
Alteromonas putrefaciens	Glucose[1] Amino acids[1,2] Lactic acid[3]	Glucose[1] Amino acids[1]	Volatile sulfides	H_2S
Brochothrix thermosphacta	Glucose1 Amino acids[2] (glutamate)	Glucose[1]	Acetic acid Acetoin Isovaleric acid Isobutyric acid	Lactic acid volatile fatty acids
Enterobacter	Glucose[1] Glucose 6-phosphate[2] Amino acids[3] Lactic acid[4]	Glucose[1] Glucose 6-phosphate[2] Mino acids[3]	 Sulfides Amines	Lactic acid CO_2, H_2 H_2S Amines
Lactobacillus	-	Glucose[1] Amino acids[2]	-	Lactic acid Volatile fatty acids

[a] Adapted from Dainty *et al.* (1985), McMeekin (1982), Lambert *et al.* (1991).
[b] The number in superscript indicates the order of utilization of this substrate.

to these organisms. When O_2 limits growth, pseudomonads attack amino acids, even when glucose is present, with the subsequent production of malodorous substances. Under anaerobic conditions, *A. putrefaciens* will generate H_2S resulting in greening of meat due to the sulfmyoglobin formation (Newton and Rigg 1979).

McMeekin (1982) stated that the growth of facultative anaerobes, *A. putrefaciens*, *Br. thermosphacta*, Enterobacteriaceae and *Lactobacillus* species is favored under reduced O_2 tension. *A. putrefaciens* uses the amino acids cysteine and serine as a substrate,under aerobic conditions, even when glucose is abundant and produces organic sulfides (Gill and Newton 1979).

Brochothrix thermosphacta is of major spoilage importance in vacuum-packaged meat containing low residual O_2. Davidson *et al.* (1968) concluded that lactic acid was the major spoilage end product of anaerobic metabolism, although small amounts of volatile acids were also formed. *Iso*-valeric and *iso*-butyric acids are also produced through the metabolism of the amino acids, leucine and valine. On the other hand Dainty and Hibbard (1980) found that acetic acid and acetoin were products of aerobic glucose metabolism, which caused the characteristic sweet off-odour, when this organism grew on beef. *Brochothrix thermosphacta* is thus of major spoilage importance in vacuum-packed meat containing low residual O_2.

The Enterobacteriaceae have a high spoilage potential, if conditions favour their growth. Under aerobic conditions, Enterobacteriaceae use glucose and glucose-6-phosphate as substrates. Some strains can produce volatile sulfides, including H_2S and malodorous amines from amino acid metabolism (Eskin *et al.* 1971, McMeekin 1982, Dainty and Mackey 1992).

Gardner *et al.* (1967) stated that *Lactobacillus* species constitute only a small proportion of the initial spoilage bacterial population of meat. When oxygen is in low concentration, as in the case in vacuum packed meats,

the developing microflora is usually dominated by *Lactobacillus* spp. (Patterson and Giggs 1978; Christopher *et al.* 1980a,b; Erichsen and Molin 1981). These fermentative organisms probably grow faster than would be competitors because they are unaffected by pH and antimicrobial products such as lactic acid, H_2O_2 and antibiotics (Hurst 1973; Newton and Gill 1978a; Gill and Newton 1980). These organisms utilize glucose for growth and produce lactic acid. When carbohydrates are exhausted, amino acids are utilized with the consequent production of volatile fatty acids which impart a "dairy" or "cheesy" odour to the vacuum-packaged meat (Nakae and Elliott 1965, Gill and Newton 1977).

Therefore the contribution of any group of microorganisms to meat spoilage depends on their initial numbers in the spoilage bacterial population, with the fastest growing species ultimately predominating. As the relative growth rates of the various groups can be affected by storage, it is therefore desirable not only to retard the total growth of all bacteria, but to modify conditions to prevent the growth of microorganisms with the highest spoilage potential (Lambert *et al.*1991).

The following review was prepared during the course of this study. It was presented at the Society for Applied Bacteriology Symposium Meeting in Edinburgh in July 1989 and published subsequently in the Society's Symposium Series. As the review is considered to be an integral part of this study, it is reproduced in the following pages.

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Staphylococci: their role in fermented sausages

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1. Introduction, 167S
2. Types of fermented sausages, 168S
3. Starter cultures, 172S
   3.1 Taxonomy, 172S
   3.2 Technological properties of starter cultures, 174S
4. Development of taste and appearance, 175S
   4.1 Flavour and aroma, 175S
   4.2 Colour, 177S
5. Public health aspects, 178S
   5.1 *Staphylococcus* as a food poisoning agent, 178S
   5.2 Factors controlling the growth and enterotoxin production of *Staphylococcus aureus*, 179S
      5.2.1 Abiotic, 179S
      5.2.2 Biotic, 180S
6. Acknowledgements, 181S
7. References, 181S

1. Introduction

Meat is not only an excellent source of nutrient (proteins, vitamins etc.) but also its high a_w (0·99) and redox status permit the growth of a wide range of organisms. The microbial spoilage of meat results primarily from the growth of undesirable organisms such as pseudomonads. Curing was probably the outcome of some of the earliest attempts to preserve meat for later consumption. The first document referring to meat curing is considered to be that of Homer. In Odyssey (about 1000–900 BC) he describes a rather primitive type of sausage as '... smoked goat-paunches filled with blood and fat offered as an hors d'oevre ...'. By the fifth century BC production of salted or cured meat products had become commonplace and the Romans had small meat shops operating under strict sanitary control (Jensen 1954). The manufacturing methods were of course—and indeed still are in some countries and areas—a craft. This was largely the case in Greece, for example, where the use of starter cultures was prohibited until recently (November, 1988) by law. Although the manufacture of fermented sausages was established in northern Europe (Hungary, Germany etc.) in the last century the microbial contributions to these products did not attract significant attention until the 1940s and 1950s. In the 1920s a few scientists suggested inoculation of sausage ingredients with nitrate-reducing micro-organisms and yeasts since the fermentation was thought to be due to the latter (Bacus 1986). At first scepticism prevailed about the use of bacteria as starter cultures. Tradition, as well as insufficent microbiological knowledge, tended to oppose the idea of possible benefits stemming from the use of starters. Today, the combined use of lactic acid bacteria and staphylococci and micrococci in meat curing has became an established feature in the meat technology of many countries. In Greece, however, the production of fermented sausages is still largely craft-based. The 'back-slop' technique, in which a part of a previous batch is used as an inoculum, is practised, especially in small-scale manufacture. There is an increasing growth in the technologically based methods used in large-scale production partly as a consequence of our membership of the European Economic Community. In this article, we review the contribution of staphylococci to sausage fermentation, a process commonly considered to be dominated by lactic acid bacteria, and consider also factors that may lead to the growth of *Staphylococcus aureus* and outbreaks of food poisoning.

Table 1. Classification of fermented sausages

Product type	Approx. weight loss during drying	Effect (a) Smoking	Effect (b) Growth of moulds	Characteristics	Examples
Dry	25–50% 35–40% 30%	– +	+ +	Cured meats; air dried, may be smoked before drying; served cold	Milano-Genoa salami, Saucisson sec, soudjouk, Pepperoni, Salami aeros, Katenrachwurst
Semi-dry	20% or 30%	+	–	Cured meats; air dried, may by smoked before drying; served cold	Summer sausage, cervelat, Lebanon bologna, porkroll, chorizos, Leukados, Beer salami, Mequez, Mortadella
Undried (fermented sausages)	10%	+	–	Fresh meats, cured or uncured stuffed, smoked, but not cooked; must be fully cooked before serving	'Teewurst', 'frische Mettwurst', countrystyle pork, kielbasa, Braunschweiger

Based on: Bacus (1986), Adams (1986) and Lücke (1985a), with additional information from Arkoudelos (unpublished) and Gokalp *et al.* (1988).

2. Types of fermented sausages

A very broad range of fermented sausages has developed over the past 2000 years (Table 1). Their popularity has shown no sign of decreasing even though refrigeration is now widely available for meat preservation. It is well known that the production of fermented sausages originated in the countries around the Mediterranean. Hungary was the first non-Mediterranean country to develop sausage production. Indeed in 1851 Piazzoni and Faddini produced the first Hungarian salami made of pure pork meat stuffed into donkey or horse intestines. This special salami was manufactured in 1896 by a company named Pick while another company (Herz) started production in 1898. Among

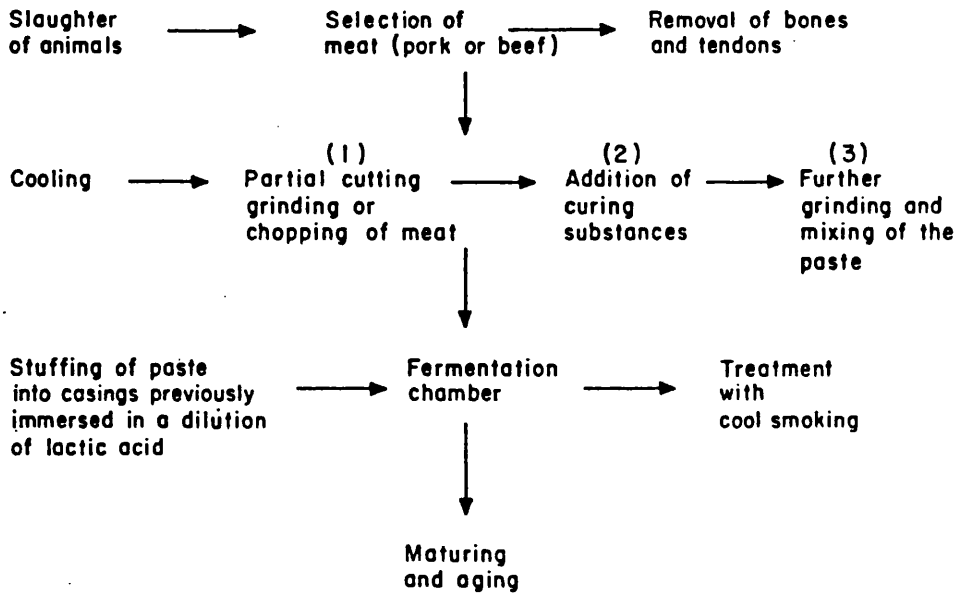

Fig. 1. Flow diagram of the processing of the Greek style fermented sausage (salami sausage).

Table 2. Formula of a typical fermented sausage mix

Ingredients	General formula	Salami aeros
Lean meat	55–70%	67·4%
Fat (lard)	25–40%	28·9%
Curing salts	3%	2·9
Fermentable carbohydrate	0·4–2·0%	0·48
Spices or flavouring	0·5%	0·4
Others (including starter culture, acidulant etc.)	0·5%	—

Based on: Bloukas & Paneras (1985) and Adams (1986).

the other European countries, the manufacture of fermented sausages commenced only 150 years or so ago. In Greece, dry and semi-dry sausages are the most common types of fermented sausages. Figure 1 shows a generalized flow diagram of the processing of Greek style sausage (salami aeros). Table 2 gives the approximate mix of a typical sausage. The type of meat can be any combination of

Table 3. Fermentation and ripening conditions of the sausages maintained in the dark

Stage of production (days)	Temperature (°C)	Relative humidity (%)	Air movement (m/s)
European style			
Fermentation			
0	24	94	
1	23	93	
2	22	92	
3	20	90	0·5–0·7
4	20	88	
5	18	86	
6	18	82–84	
Ripening	13–15	70–80	0·05–0·1
American style			
Fermentation			
1–2	27–37	90	—
Ripening	10–11	68–72	—

Based on Bloukas & Paneras (1985) and Bacus (1984).

Table 4. Composition of some commercial dry sausages

Product type	Moisure (%)	Fat %	NaCl %	pH	a_w
Lebanon bologna (5)*	58·92	15·12	3·53	4·42	—
Dry salami (8)	36·21	33·7	4·54	4·9	—
Salami, Belgian and German type (34)	33·6	41·8	3·5	—	—
Pepperoni (8)	25·0	42·0	—	5·1	0·84
Greek style (10)	35.0	—	—	4·9	0·75
Salami aeros (9)	32—36	—	—	4·8–5·15	—

Based on: Acton & Dick (1976); Palumbo *et al.* (1976a, b); Bacus (1986); Arkoudelos (unpublished); Bloukas & Paneras (1985).

* No. of samples analysed in parenthesis.

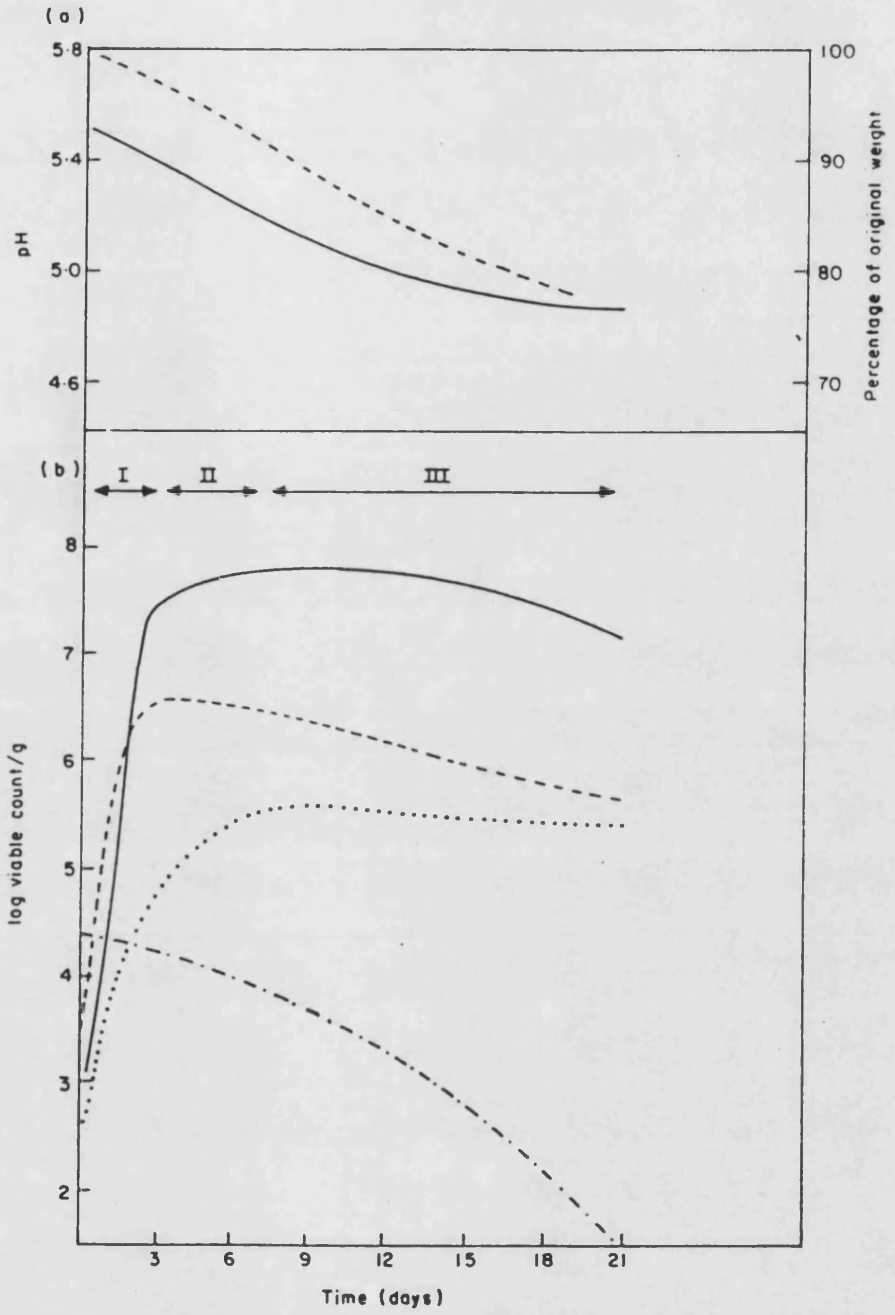

Fig. 2. Development of the microflora (log cfu/g fresh weight) during ripening of salami-type sausage by the 'natural' method (use of nitrate, low amounts of sugar, low fermentation temperature). Ripening conditions: I. 18–20°C; II. 20–22°C, smoke; III. 15–17°C. (a) ———, pH; – – – –, weight. (b) ———, lactobacilli; – – – –, Micrococcaceae; ·····, streptococci; –·–·–·–, Gram-negatives. Reproduced with permission from Lücke, F.K. (1985) Fermented sausages. In *Microbiology of Fermented Foods* Vol. 2, ed. Wood, B.J.B. London: Elsevier Applied Science.

beef or pork. Eating habits, religious traditions and meat prices determine the choice of meat. The production of fermented sausages started in islands such as Leukas and Mykonos (Katsaras 1987). Fermented sausage production in many areas is still based on the traditional way of the 'back-slopping technique'. In other aspects the fermented sausages industry in Greece is well developed and equipped. The import of 'know-how', especially from Germany, and the use of guidelines of Good Manufacturing Practices ensure a good product. From a technological point of view, the suitability of a meat for fermented sausage production depends on its pH, its water-holding capacity—'Pale Soft

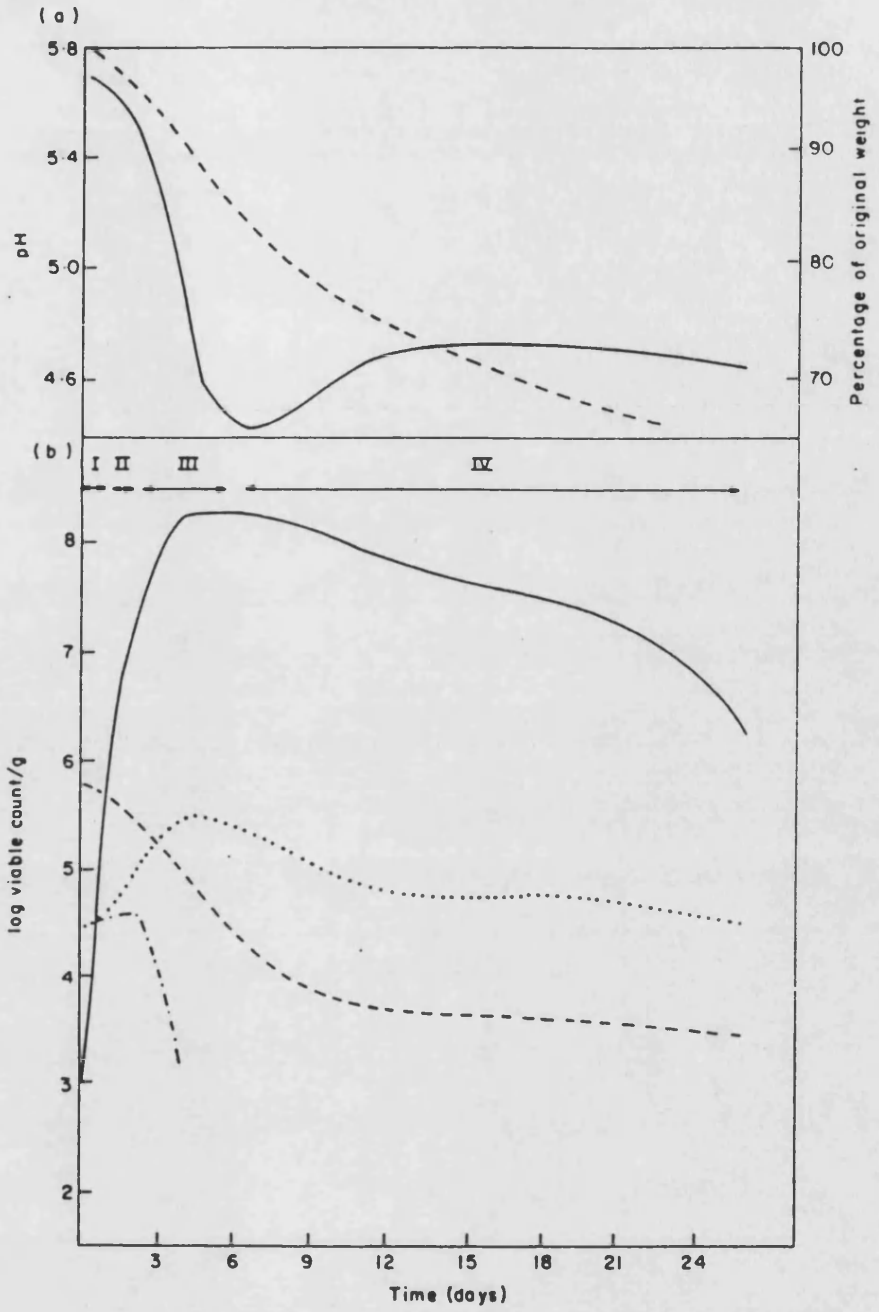

Fig. 3. Development of the microflora (log cfu/g fresh weight) during ripening of salami-type sausage by the 'rapid' method (use of nitrite, high amounts of sugar and elevated fermentation temperature). Ripening conditions: I. 4°C; II. 18°C; III. 24°C, smoke; IV. 18–20°C. (a) ———, ph; – – – –, weight. (b) ———, lactobacilli; – – – –, Micrococcaceae; ·····; streptococci; –·–·–·, coliforms. Reproduced with permission from Lücke, F.K. (1985) Fermented sausages. In *Microbiology of Fermented Foods* Vol. 2, ed. Wood, B.J.B. London, Elsevier Applied Science.

Exudative' (PSE) meat is preferred to 'Dark Firm Dry' (DFD) meat—and on the desired intensity of the colour of the finished product (beef has a more intense colour than pork). Meat from old animals is preferred by butchers for dry sausage manufacture. The addition of the appropriate carbohydrates (e.g. glucose) and the use of a starter culture seem to be of equal importance to that of meat quality in sausage production (Bacus 1986). It is well known that pseudomonads are the dominant organisms on chill-stored meat (Gill 1982). When the meat is processed into a raw sausage, the water activity is reduced to 0·96–0·97 as a consequence of the addition of salt and the oxygen trapped in the mixture is rapidly consumed. These changes reduce the competitiveness of Gram-negative organisms (Gill

Table 5. *Staphylococcus* spp. isolated from jointed or minced meat (beef or pork), poultry and their products

Strain	Meat and poultry	Fermented sausages	References
Staphylococcus	+	+	11, 14, 15, 20–22, 28
aureus	+	+	3, 5–10, 12–16, 17–19, 23–25
xylosus		+	1, 2, 8, 29
haemolyticus		+	27
saprophyticus		+	1, 26, 27, 29
warneri		+	1
caprae		+	1
carnosus		+	2, 4
simulans		+	26
capitis		+	27
epidermidis		+	27, 29

References: 1. Seager *et al.* (1986); 2. Blackburn (1985); 3. Reali (1982); 4. Schleifer & Fischer (1982); 5. Gibbs *et al.* (1978); 6. Hintlan & Hotchniss (1987); 7. Wieneke (1974); 8. Emswiller-Rose *et al.* (1980); 9. Warburton *et al.* (1987); 10. Gokalp *et al.* (1988); 11. Martinez *et al.* (1984); 12. Robbs & Robbs (1979); 13. Hartog *et al.* (1987); 14. Pullen & Genigeorgis (1977); 15. Jay (1961); 16. Jay (1962); 17. Eddy & Ingram (1962); 18. Canale-Parola & Ordal (1957); 19. Minor & Marth (1972ab; 20. Geschey & Neira (1982); 21. Adams & Mead (1983); 22. Bryan (1976); 23. Genigeorgis *et al.* (1980); 24. Barber & Deibel (1972); 25. Bacus & Brown (1981); 26. Fischer & Schleifer, (1980); 27. Simonetti & Cantoni (1983); 28. Yang *et al.* (1988); 29. Delarras (1982).

1982; Mitchell & Dawes 1982), and lactic acid bacteria, staphylococci and micrococci are enriched (Bacus 1986). This is illustrated in Fig. 2 which shows the development of the microflora in fermented sausage produced by the traditional (natural) method. With the 'rapid' methods (Fig. 3), which are used mainly by the American industry, the conditions are such that the pseudomonads and coliform organisms die out, lactobacilli dominate the microflora within 3 d, and micrococci make a negligible contribution. In Europe, the adoption of the 'rapid' method has been less marked than in America. This is because in Europe the microbial conversion of nitrate to nitrite is preferred to the addition of NO_2 only. The 'natural' process also contributes to the flavour of the dried products as will be discussed below. In general, European-style sausages are fermented at lower temperatures (Table 3) with a slower fermentation rate compared with those of America. In Table 4 the composition of some commercial dry sausages made by both methods are shown.

3. Starter cultures

3.1 TAXONOMY

Although it is now generally recognized that strains of *Micrococcus* and *Staphylococcus* are involved in the fermentation of at least some types of fermented sausages, the taxonomy, occurrence and the contribution of these organisms is not always discussed in detail in the relevant literature. There is still a tendency by authors to use 'micrococci' or Micrococcaceae in preference to specific names. Indeed even these names were omitted from a recent review (Smith & Palumbo 1983). The contribution of Pohja & Gyllenberg (1962) to a meeting organized by the Society of Applied Bacteriology (in London, 1962) can be taken as a starting point of studies which have led to our current understanding of the role of staphylococci in sausage production. Niinivaara & Pohja (1956, 1957a, b) and Pohja & Gyllenberg (1962) showed that such organisms grew extensively during the fermentation phase of sausage production and contributed to the organoleptic properties of the product. Although they applied the emerging techniques of numerical taxonomy to the strains they isolated, a satisfactory

Table 6. Bacteria used as starter cultures for fermented meat products (cured)

Organisms	References
1. Lactobacillaceae	
Lactobacillus	
plantarum	1–6, 10–13, 18, 22–24, 27, 28, 34, 39–44
LP-1; TF-1; -4;	17
acidophilus	4, 5
casei	5
fermenti	5
brevis	4–6
buchneri	5
carvatus	5, 8, 23
Lc5, Lc2	7
sake	2, 4, 5, 8, 23
LS9	7
delbruecki	5
farciminis	4
hispanicus CP-9	4
specialis CP-2P	4
CP-26	4
lucanicatium	4
cucumeris	4
pentosus	4
rabinosus	4
leichmannii	4
pentoaceticus	4
wehmeri	4
lycopersica	4
gayoni	4
mannitopeous	4
fermentum	4
L110	4
NRRL-B-5632	4
822	4
4669/6	4
Pediococcus	1, 5, 6, 9, 14, 16, 27, 32, 33, 36–40, 42, 44, 45
cerevisiae NRRL-B-562; CPO 18; PS-23; -1; Ac-1;	4, 11, 15, 26
acidilactici	5, 6, 10, 25, 29, 30, 34, 35
pentosaceus PC-39	4–6, 19–22, 25
PC-30	4
Streptococcus (*Lactococcus*)	
lactis	5
diacetylactis	5
acidilactici	5
Micrococcus	
aurantiacus M35	3–5, 31
candidus	4, 5
varians	4, 5, 28
epidermis	4, 5
conglomeratus	4, 5
aqualitis	5
kristinae	5
lactis	5
caseolyticus	4, 5
violagabriola	22
199/10	4
specialis	
MF-1; M17; MIII; DB-6	4
aquatis	4
P4	4
M104	4
M86	4

Table 6—*continued*

Organisms	References
Staphylococcus	
carnosus	2, 4, 5
caseolyticus	5
xylosus	4, 5
simulans	
MIII; M17; MIII; M17	4, 5, 43
saprophyticus	5

References: 1. Walsh & Hoover (1987); 2. Lücke (1985a); 3. Nurmi (1966); 4. Liepe (1982); 5. Bacus (1986); 6. Smith & Palumbo (1983); 7. Kneibler *et al.* (1986); 8. Lucke *et al.* (1986); 9. Keller & Acton (1974); 10. Smith & Palumbo (1978b); 11. Kato *et al.* (1985a); 12. Numata *et al.* (1988a); 13. Joseph *et al.* (1978); 14. Eitenmiller *et al.* (1978); 15. Klement *et al.* (1974); 16. Deibel *et al.* (1961a); 17. Numata *et al.* (1988b); 18. Lee & Song (1987); 19. Lee (1987); 20. Tetlow & Hoover (1968); 21. Blickstad & Molin (1981); 22. Olsen (1985); 23. Lucke (1985b); 24. Nes & Skjelkale (1982); 25. Raccach (1987); 26. Kato *et al.* (1986a); 27. Deibel *et al.* (1961b); 28. Nestorov *et al.* (1985); 29. Acton & Dick (1977a); 30. Acton & Dick (1977b); 31. Niinivaara (1955); 32. Deibel & Niven (1957); 33. Wardlaw *et al.* (1973); 34. Zaika & Kissinger (1984); 35. Acton *et al.* (1977); 36. Townsend *et al.* (1980); 37. Niskanen & Nurmi (1976); 38. Townsend *et al.* (1983); 39. Donelly *et al.* (1982); 40. Zaika *et al.* (1976); 41. Trevor *et al.* (1983); 42. Zaika & Kissinger (1979); 43. Simonetti *et al.* (1983); 44. Zaika *et al.* (1978); 45. Everson *et al.* (1970a, b).

Table 7. Contribution of different starters cultures to the organoleptic properties of dry sausages

Organism	Lactic acid production*	Lipolysis†	Proteolysis
Pediococci	1 (1)	3 NF	2‡ NF
Lactobacilli§	2 (1)	2 NF	2‡ NF
Micrococci-type§	3 (2)	1	1‡

* Kandler (1983); Olsen (1985); Hadziosmanovic *et al.* (1979); Sanz *et al.* (1988).

† Reuter (1975); Delarras (1982); Bacus (1986); Nurmi & Niinivaara (1964); Smith & Alford (1968); Giolitti *et al.* (1971); Demeyer *et al.* (1974).

‡ Klement *et al.* (1974); Sajber *et al.* (1971).

§ Synergetic effects (Andres 1977).

NF, the micro-organisms did not possess proteolytic or lipolytic properties according to Nordal & Slinde (1980), Deibel *et al.* (1961a, b). These results are not in agreement with findings of Reuter (1975) and Law & Kolstad (1983).

classification was not achieved. Indeed such a situation was not attained until the studies of Reinbaben & Hadlock (1979) and Fischer & Schleifer (1980). These workers noted the occurrence of staphylococci in sausages made in Germany and identified their strains with *Staph. somulans*. Subsequently the organisms were assigned by Schleifer & Fischer (1982) to a new species, *Staph. carnosus*. This organism was isolated occasionally by Seager *et al.* (1986) in a survey of fermented sausages imported into the UK. We have isolated it from Greek sausages also (Arkoudelos, unpublished). Moreover *Staph. xylosus*, *Staph. warneri*, *Staph. caprae*, and *Staph. saprophyticus* were also isolated (Table 5) by Seager *et al.* (1986).

3.2 TECHNOLOGICAL PROPERTIES OF STARTER CULTURES

As discussed previously, microbiological studies of meat fermentation have developed to such an extent that modern technology using advanced production methods depends upon the use of particular bacteria rather than the enrichment of meat organisms to ensure successful manufacture. A very

large number of species have been tested for this purpose (Table 6). Judging from the literature, however, only a few have been adopted for common use. These selected strains carry out specific modifications of substrates under controlled conditions (Liepe 1978). Table 7 shows the three important technological characteristics (glycolysis, lipolysis and proteolysis) of the different starter cultures. In general, the following are used today as starter cultures in meat fermentation; *Lactobacillus* (Nurmi 1966; Everson *et al.* 1970a; Bacus 1986; Weber 1986), *Pediococcus* (Everson *et al.* 1970a; Smith & Palumbo 1983; Raccach 1987) and *Staphylococcus* and *Micrococcus* (Andres 1977; Bacus & Brown 1981; Simonetti *et al.* 1983; Adams 1986). The first two genera are homofermentative; lactic acid is the main end product of glucose metabolism with acetic acid, acetoin and carbon dioxide as minor products. These obligatory fermentative organisms are catalase negative and some strains produce antimicrobial compounds (Raccach 1987). Pediococci have been used in preference to lactobacilli in the past for two main reasons; first, all members of this genus are homofermentative (Raccach 1987) and second their resistance to lyophilization is greater than that of *Lactobacillus* (Adams 1986). Indeed lyophilized and frozen concentrates of pediococci are commonly used in sausage production (Everson *et al.* 1970a, b; Raccach 1987). Members of the genera *Staphylococcus* and *Micrococcus* (Table 6) are used as starter cultures because of their ability to reduce nitrate and produce catalase (Lücke 1985a, b; Bacus 1986). In his review Bacus (1986) reported that micrococci-type bacteria have been used extensively for many years but solely in European sausages. In general the proteolytic activity of lactic acid bacteria is weak (Kitchell & Shaw 1975; Law & Kolstad 1983). The spoilage of meat due to proteolysis or, more important, the development of aroma and flavour with free amino acids (FAA) and free fatty acids (FFA) in the case of fermented sausages, cannot be attributed to this activity. Although micrococci/staphylococci produce extracellular proteinases (McDonald & Chamber 1966; Donham *et al.* 1988), their role in the proteolysis of fermented sausages is in dispute. The increase of FAA and the proteolysis in fermented sausage has been attributed to micrococci (Sajber *et al.* 1971; Bacus 1986; and a trade leaflet of Chr. Hansens Co). Although Niinivaara *et al.* (1964) did not find any differences in FAA between sausages fermented naturally or inoculated deliberately with micrococci, they suggested that the decomposition of proteins during the fermentation is the result of the activity of inoculated bacteria. In contrast, Giolitti (1960; cited by Sajber *et al.* 1971) is of the opinion that the proteolytic activity is a property of the meat, the microorganisms being of little importance. The production of FAA by the lipolytic enzymes under aerobic (*Micrococcus*) and aerobic/anaerobic conditions (*Staphylococcus*) (Smith & Alford 1968; Simonetti & Cantoni 1983), however, has been considered to contribute to sausage flavour and/or aroma production.

4. Development of taste and appearance

4.1 FLAVOUR AND AROMA

The fermentation of dry sausage has been represented as a set of overall reactions involving carbohydrate, protein and lipid degradation and as a set of simple equations characterizing the formation of end-products (Demeyer & Verplaeste 1985). It is well known that during the *post-mortem* period a small portion of the glycogen in meat is converted to glucose and finally to lactic acid (Lawrie 1974). The glucose is utilized directly or converted by pseudomonads to gluconate. The latter is used subsequent to glucose exhaustion when meat spoils at chill temperature (Gill 1982; Nychas *et al.* 1988). Once both these substrates are exhausted, many aerobic or faculatively anaerobic bacteria begin to attack amino acids which serve as an alternative carbon and energy source under either aerobic or anaerobic conditions (Gill 1982; Nychas 1984; Nychas & Arkoudelos unpublished). As the glucose content of fresh meat (beef or pork) is of the order of 50–200 mg/100 g meat (Lawrie 1974; Farber & Idziak 1982; Nychas 1984), microbial action results in only a small change in pH. Fermentable carbohydrate must therefore be added to meat intended for the production of fermented sausages. The variety and the amount of carbohydrate added are crucial because they determine the rate and extent of lactic acid formation and the composition of the sausage microflora (Acton *et al.* 1977; Klettner & List 1980; Lücke 1985a, b; Olsen 1985; Lee 1987). Indeed, these workers found that

glucose and sucrose caused similar rates of pH decrease during the fermentation of sausages with *Ped. acidilactici*, *Lact. plantarum* and *Ped. pentosaceus*. A slower rate of pH decrease obtained with lactose. Moreover, Olsen (1985) showed that the rate of the decrease of pH did not differ significantly when glucose was added over the range 0·1%–1% in sausages. Pediococci and lactobacilli, the major members of the fermentation flora, ferment glucose and lactose via the Embden–Meyerhof (EM) pathway (Kandler 1983; Garvie 1984; Tetlow & Hoover 1988) while micrococci-type bacteria (e.g. micrococci, staphylococci) use both the EM and hexose monophosphate pathway (HMP) (Strasters & Winkler 1963; Blumental 1972). Lactic acid, the main contributor to the 'tangy' flavour of sausages, is the principal product of metabolism of these carbohydrates during the fermentation and/or ripening of sausage, as would be expected from the dominance of homofermentative micro-organisms in the meat. The ratio of the two enantiomers (*d*, *l*-lactic acid) varies somewhat and depends on the species of the bacteria present in fermented sausages (List & Klettner 1978; Lücke 1985a) or in broth culture (Blickstad & Molin 1981). Ethanol and carbon dioxide, in equal portions, are produced by hetero-fermentative micro-organisms. When the 'bifidum pathway' operates in these bacteria, lactate and acetate are produced in a ratio 2 : 3 (Gottschalk 1979). Fermentation products other than lactic acid, such as acetic acid, ethanol, acetoin, carbon dioxide, pyruvic acid etc., are produced in various amounts during sausage fermentation (Strasters & Winkler 1963; Blumental 1972; Vandekerchove & Demeyer 1975; Demeyer 1982; Thornill & Cogan 1984; Tetlow & Hoover 1988; Arkoudelos, unpublished) and in broth cultures (Blickstad & Molin 1981). Table 8 shows the major end-products formed during the fermentation and ripening period of sausages. The end-product formation depends upon the composition and the size of inoculum of starter culture (Klement *et al.* 1974; Eitenmiller *et al.* 1978; Hadziosmanovic *et al.* 1979; Olsen 1985; Kato *et al.* 1985; Bacus 1986; Numata *et al.* 1988a. b), the type and the amount of carbohydrate added (Deketeleare *et al.* 1974; Acton *et al.* 1977; Olsen 1985; Lee 1987; Lois *et al.* 1987), the source and previous treatment of meat proteins (Klement *et al.* 1973; Townsend *et al.* 1980; Pezacki & Pezacka 1987), and the addition of various additives such as

Table 8. End products formed during fermentation and ripening of dry fermented sausages

Product	Natural flora	Starter culture	Micrococcus added
Lactic acid	1, 2, 5, 14, 23, 26	2, 3, 11, 16, 20, 22–24, 27	11, 22
Acetic	1, 4, 5, 26	11, 20, 27	11
Diacetyl	11, 18	11, 27	11
Acetoin	11, 18	11, 27	11
2, 3-Butyleneglycol	18		
Ethanol	7, 11, 18	11	11
Soluble proteins	1, 2, 6, 9	2, 6, 9, 19	
Ammonia	6, 9, 11, 13, 23, 26	6, 9, 11, 15, 23	11
Butyric	2, 5, 11	2	
Oxalic	9		
Citric	4		
Pyruvic	4		
Malic	4		
Formic	4, 5, 11		
Fumaric	4		
Propionic	2, 4, 5, 11	2, 11	
Free fatty acids	6, 8, 13, 17	6	
Amines	9, 10, 12, 14, 25, 28	10, 9	
Free amino acids	1, 6, 9, 23	6, 9, 15, 19, 21, 23	11, 19
Aldehydes, ketones	5		

References: 1. Demeyer (1982); 2. Deketelaere *et al.* (1974); 3. Acton *et al.* (1977); 4. Virgili & Parolari (1986); 5. Halvarson (1973); 6. Numata *et al.* (1988a); 7. Pezacki & Szostak (1962); 8. Demeyer *et al.* (1974); 9. Dierick *et al.* (1974); 10. Eitenmiller *et al.* (1978); 11. Arkoudelos (unpublished data); 12. Vanderkerckhove (1977); 13. Lois *et al.* (1987); 14. Santos-Buelga *et al.* (1981, 1986); 15. Lee & Song (1987); 16. Joseph *et al.* (1978); 17. Cantoni *et al.* (1964); 18. Pyrcz & Pezacki (1975); 19. Sajber *et al.* (1971); 20. Kneibler *et al.* (1986); 21. Eskeland & Nordal (1980); 22. Olsen (1985); 23. Demeyer & Verplaetse (1985); 24. Keller & Acton (1974); 25. Rice *et al.* (1976); 26. Demeyer *et al.* (1979); 27. Raccach (1987); 28. Kohler & Eitenmiller (1978).

glucono-delta-lactone (GDL), spices etc. (Nes & Skejelvale 1982; Lee 1987). Although lactic acid bacteria isolated from meat possess weak lipolytic and proteolytic systems, an attribute noted above, the contribution of these enzymes to flavour is uncertain. It is known that many staphylococci and micrococci isolated from fermented sausages actively attack pork fat (Nurmi & Niinivaara 1964; Cantoni *et al.* 1967; Smith & Alford 1968; Alford *et al.* 1971; Demeyer *et al.* 1974; Delarras 1982; Brankova 1985; Bacus 1986). Indeed most species of micrococci demonstrate lipolytic activity under aerobic conditions (Simonetti & Cantoni 1983). In commercial establishments different strains of micrococci are preferred; the selection is based on the specific lipolytic activity and the extent of volatile fatty acids (VFA) formation of a strain (Cattaneo *et al.* 1983; Simonetti *et al.* 1983). Both staphylococci and micrococci are catalase positive (Brankova 1985). This enzyme can be an effective antioxidant which retards oxidative rancidity.

4.2 COLOUR

Myoglobin is the most abundant pigment in muscle (Lawrie 1974) and its presence is mainly responsible for the desirable/undesirable colour of meat. Both myoglobin and haemoglobin are haem proteins which consist of a protein (globin) complexed to haem, with an iron nucleus attached to a porphyrin ring (Kramlich *et al.* 1973). The reactions of these haem pigments of meat, myoglobin and haemoglobin, are important in determining the final colour of fresh and cured meats (Fox 1966). After slaughter, meat usually turns brown because of the low oxygen supply (Fox 1966). Nitrite/nitrate are added to meat products for aesthetic reasons. In the presence of nitrite/nitrate, a number of different pathways can lead to the development of the pigment present in cured meat (Fig. 4). Basically nitric oxide, which is produced by the reduction of nitrate/nitrite, reacts with the myoglobin to form nitrosylmetmyoglobin (Lee & Cassens 1976; Cassens *et al.* 1979). With further treatment with heat/and or smoke, a stable pink pigment is formed (nitrosohemochromogen). Although the addition

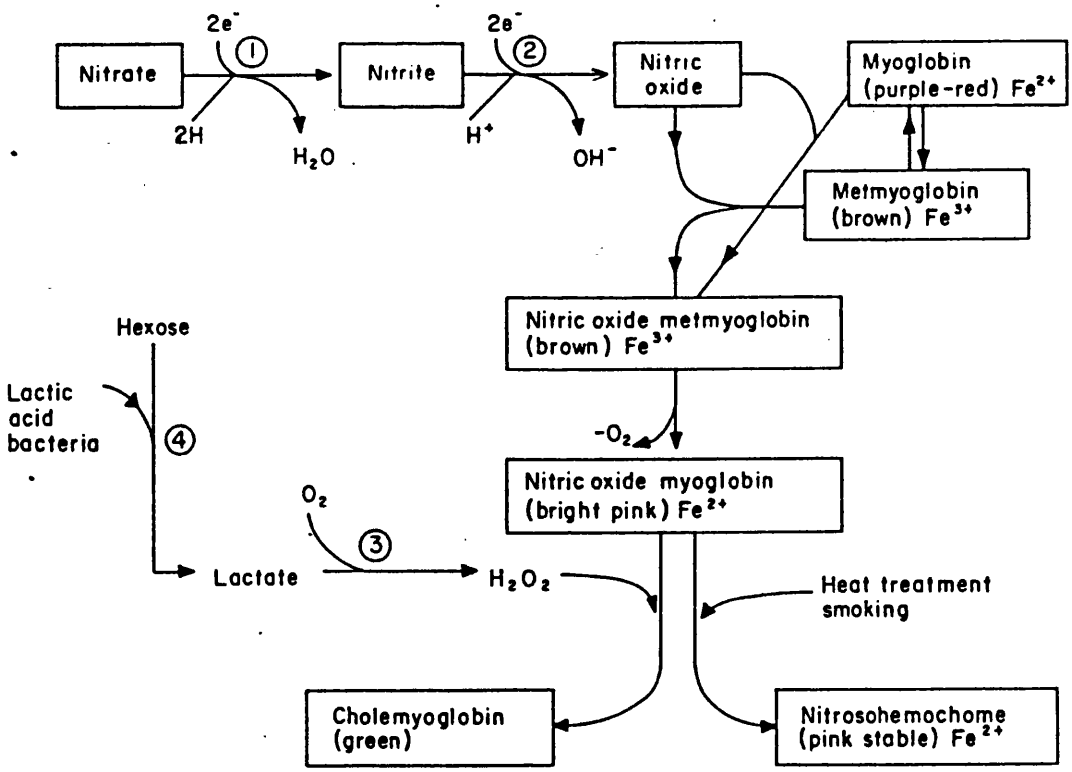

Fig. 4. Diagram showing the changes of the heme pigments colour during the fermentation of sausages. 1. Nitrate reductase; 2. nitrite reductase; 3. oxidases; 4. enzymes of the Embden-Meyerhof-Parnas pathway. Based on Fox (1966), Liepe (1983), Kandler (1983), Gray & Pearson (1984) and Cassens *et al.* (1979).

of nitrate/nitrite accelerates the formation and contributes to the stability of the red cured colour and flavour of the cured meat (Hadden *et al.* 1975; Lücke 1985a), they have one crucial disadvantage: the possibility that carcinogenic nitrosamines are formed and/or the possible carcinogenic activity of nitrite *per se* (Sebranek 1979). For this reason the presence of nitrate/nitrite reducing micro-organisms is also crucial not only for the formation and stability of the cured meat colour but also for reduction of nitrate via nitrite to dinitrogen monoxide, with the final product elementary nitrogen (Liepe 1983; Gray & Pearson 1984; Lücke 1985a, b). With the exception of *Lact. plantarum* (Smith & Palumbo 1978b), staphylococci and micrococci are the predominant organisms in this reduction (Niinivaara & Pohja 1957a, b; Liepe 1983; Fischer & Schleifer 1980; Lücke 1985a, b; Lücke *et al.* 1986). Indeed, both groups of organisms produce nitrate reductase (EC 1.6.6.1–3) and nitrite reductase (EC 1.6.6.4) (Liepe 1983; Brankova 1985). Both enzymes would account for the removal (Fig. 4) of residual nitrates (Niinivaara 1955; Simonetti & Cantoni 1983; Cattaneo *et al.* 1983; Bacus 1986; Lücke *et al.* 1986) (Fig. 4). Starter cultures or raw ingredients (Gardner 1983) may contain lactobacilli; these bacteria can cause a discoloration of cured meat by the formation of peroxide (Niven *et al.* 1954; Bacus 1986). It is well known that many lactobacilli produce H_2O_2 (Raccach & Baker 1978; Kandler 1983; Lücke *et al.* 1986). The H_2O_2 is formed by the oxidation of lactate (Hochst 1979). The lactobacilli are divided in two groups. One possess a flavin-containing *l*-lactate oxidase using O_2 as electron acceptor, the other requires an acceptor methylene blue (MB) to perform lactate oxidation at significant rates. In group I (*Lact. curvatus, Lact. sake, Lact. acidophilus, Lact. bulgaricus, Lact. lactis*) lactate oxidation with O_2 yields H_2O_2 and pyruvate. Group II strains (*Lact. plantarum, Lact. casei, Lact. coryniformis*) exhibit only a very low rate of lactate oxidation to acetate and carbon dioxide which was increased tenfold by the addition of MB—when H_2O_2 and pyruvate are produced, the accumulation of the latter in both cases leads to spontaneous breakdown of the acid (Kandler 1983). Lactobacilli are more resistant to peroxides than many other bacteria in fermented sausages (Lücke 1985a). Catalase has been found to counteract peroxide formation (Gottschalk 1979). Thus synthesis of catalase is advantageous in sausage production (Rozier 1971). As staphylococci and micrococci possess this enzyme, their use in meat curing offer advantages over starter cultures of lactic acid bacteria (Liepe 1983; Lücke 1985a, b). Moreover, it is well known (Liepe 1983) that catalase also inhibits rancidity. In general the contribution of lactobacilli and pediococci is evident during the first stages of fermentation and ripening whereas the contribution of staphylococci and micrococci is related to changes affecting the flavour, aroma and stability of colour of the final product.

5. Public health aspects

5.1 *Staphylococcus* AS A FOOD POISONING AGENT

Staphylococcoccus aureus is of significant practical importance on meat. Not only can this bacterium (Tables 5 & 9) cause a variety of infections in meat animals, as well as in humans, but it is also the causative agent of a major form of food poisoning. See, e.g., Mend & Dodd pp. 81S–91S and Gilmour & Harvey pp. 147S–166S of this Symposium. For example, in the USA during the period 1975–9, 540 food poisoning outbreaks were reported, with *Staph. aureus* responsible for 28% (153 outbreaks). Misuse of foods in food service operations seems to be the major cause of outbreaks, followed by mishandling in the home. Only a few outbreaks appear to be directly attributed to contamination during food processing operations (Smith *et al.* 1983; Genigeorgis 1987). *Staphylococcus aureus* is predominantly parasitic on man, the nose being the main site for multiplication. Staphylococci also survive on the skin and are found in the faeces (Minor & Marth 1972a; Wieneke 1974; Reali 1982). As these pathogens are also carried by animals, transfer of the infection—although rare—to man can occur. The occurrence of *Staph. aureus* in fermented sausages has been reported on many occasions (Scheusner & Harmon 1973; Pullen & Genigeorgis 1977; Robbs & Robbs 1979; Neira 1982; Martinez *et al.* 1984; Hartog *et al.* 1987; Warburton *et al.* 1987; Gokalp *et al.* 1988), at various stages of sausage production as well as in the finished product in retail shops. Bad practices during manufacture, especially poor conditions of fermentation and poor conditions of storage, have lead to food-

Table 9. Staphylococci isolated from animals

Species	References
Staphylococcus	
arlettae	1
equorum	1
kloosii	1
lentus	1
gallinarium	1
aureus	2–5
epidermidis	6

References: 1. Schleifer *et al.* (1984); 2. Gibbs *et al.* (1978); 3. Devriese *et al.* (1972); 4. Devriese *et al.* 1975; 5. Adams & Mead 1983; 6. Devriese & Oeding (1975).

poisoning outbreaks from products, especially those that are consumed raw (Baran & Stevenson 1975; Pullen & Genigeorgis 1977; Bacus & Brown 1981; Hartog *et al.* 1987). Moreover, Williams *et al.* (1983) examined 111 samples from randomly selected meat processing establishments. They found that *Staph. aureus* was always present on cutting tables, on bandsaws, grinders and knives. In a similar study in Greece, Grigoriadis *et al.* (1985) reported that *Staph. aureus* was always found on wooden cutting tables. When compared with other foods, fermented sausages have a good safety record (Minor & Marth 1972a, b, c; Wieneke 1974; Roberts *et al.* 1980; Stiles & Ng 1981; Reali 1982; Adams & Mead 1983; Hudson *et al.* 1986; Yang *et al.* 1988). Even so, a food prepared by fermentation will afford opportunities for the growth of pathogens should the fermentation be delayed. The occasional outbreaks of *Staph. aureus* food poisoning, which appear to be more common in the USA (Lücke 1985a; Adams·1986) and Canada (Warburton *et al.* 1987) than in Europe (Lücke 1985a; Adams 1986), have led to studies of the ability of *Staph. aureus* to grow in fermented sausages (Pullen & Genigeorgis 1977; Metaxopoulos *et al.* 1981a, b). Illness results from the ingestion of a water-soluble, heat-stable enterotoxins (Minor & Marth 1972a, b) secreted by the staphylococcal cells. Secretion of these enterotoxins occurs at different periods of the growth phase, either as primary or secondary metabolites (Minor & Marth 1972a).

5.2 FACTORS CONTROLLING THE GROWTH AND ENTEROTOXIN PRODUCTION OF *Staphylococcus aureus*

5.2.1 Abiotic

Growth and enterotoxin production by *Staph. aureus* is influenced by many factors (Table 10). The slight inhibitory action of NaCl on *Staph. aureus* has been attributed to a decrease in the a_w (Sofos 1983; Martinez *et al.* 1986) or to inhibition of a number of biochemical systems (Smith *et al.* 1987). Although *Staph. aureus* is tolerant of low a_w (Troller 1971), its growth is retarded by elevated concentrations of NaCl (Lechowich *et al.* 1956; McLean *et al.* 1968; Genigeorgis *et al.* 1971a; Metaxopoulos *et al.* 1981a, b; Hurst & Hughes 1983; Marcy *et al.* 1985) and enterotoxin production may be inhibited (Genigeorgis & Sadler 1966; Hojvat & Jackson 1969; Genigeorgis *et al.* 1971a, b; Minor & Marth 1972b; Smith *et al.* 1983, 1987). The combined effects of NaCl, a_w and pH drastically affect the growth of *Staph. aureus* in fermented sausages. Indeed Troller (1971), Genigeorgis *et al.* (1971a, b, c), Boylan *et al.* (1976), Metaxopoulos *et al.* (1981a, b) and Martinez *et al.* (1986) found that a rapid decrease in pH—a feature of a good fermentation—with a relative high concentration of NaCl and concomitant low a_w (*ca* 0·90–0·92) inhibited not only the growth of *Staph. aureus* but also the production of the enterotoxins A, B, and C. Metaxopoulos *et al.* (1981a, b) suggested that the lack of detectable levels of enterotoxin production might be due to the combined effect of pH, NaCl, a_w and

Table 10. Factors affecting growth and toxin production of *Staphylococcus aureus*

Environmental	
Abiotic	Biotic
a. Physico-chemicals	1. Type and size of initial inoculum of starter cultures
1. pH	2. Competition with other indigenous organisms such as coliforms, yeasts, *Proteus* spp., *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus faecalis*
2. a_w	
b. Additives	
1. Type and amount of carbohydrates	
2. Nitrates/nitrites	
3. Glucono-delta-lactone	
4. NaCl	
5. Proteins	
6. Spices	
c. Conditions applied during the process of fermented sausages	
1. Oxygen tension	
2. Temperature	
3. Humidity	

nitrate. Nitrate has been found to affect toxin production and, in high concentrations, the growth of *Staph. aureus per se* (Lechowich *et al.* 1956; McLean *et al.* 1968; Labots 1976; Bacus & Brown 1981; Martinez *et al.* 1986). It was found that the nitrate effect was more pronounced under anaerobic conditions in dry sausages (Lechowich *et al.* 1956; Barber & Deibel 1972). The growth of *Staph. aureus* in the surface layer of brine-fermented sausage is said to be retarded and more affected by the usual amounts of nitrite than growth in the surface layer of air-fermented sausages (Labots 1976). This author noted that small amounts of nitrite were very effective in preventing staphylococcal growth in the core (anaerobic conditions) of the sausages, though nitrite was a more effective inhibitory agent in the core of brine-fermented than in that of air-fermented sausages. Analogous results with low oxygen tension have been reported for *Staph. aureus* growth and toxin production—in broth and meat products such as fermented sausages (McLean *et al.* 1968; Baird-Parker 1971; Barber & Deibel 1972; Dietrich *et al.* 1972; Coleman 1985; Stersky *et al.* 1986). Indeed Smith *et al.* (1983) state that the alteration of atmospheric composition can influence the potential for enterotoxin production, particularly when oxygen is eliminated or its concentration reduced. The addition of GDL (a labile acidulant used to decrease the pH of raw sausage; Daly *et al.* 1973; Metaxopoulos *et al.* 1981a, b), smoking (Tatini *et al.* 1976a, b; Donelly *et al.* 1982), concentration of proteins (Tatini *et al.* 1976b) or additives such as monosodium glutamate in soy sauce (Hurst & Hughes 1983) and polyphosphates (Chung-Mei & Shelef 1986) have also been reported to influence the growth of *Staph. aureus*. In one study the addition of proteins appeared to enhance toxin production (Tatini *et al.* 1976b). Inhibition of the growth of pathogens such as *Staph. aureus* by other species is difficult to interpret. Inhibition may be caused by antimicrobial agents *per se* or enhancement of growth of competing organisms, particularly the starter cultures by trace metals (Zaika & Kissinger 1969; Wilkins & Board 1989). Of all the factors considered so far, temperature is the most crucial one. Tatini *et al.* (1976a, b) found that growth of *Staph. aureus* occurred within a range of 7 to 47·8°C with growth optimum at 37°C. The corresponding values for enterotoxin production were in a range of 10°C to 46°C. Generally it was found that an increase in the incubation temperature increased both the growth rate and the toxin production of *Staph. aureus* (McClean *et al.* 1968; Hojvat & Jackson 1969; Smith *et al.* 1983).

5.2.2 Biotic

As has been mentioned earlier, food poisoning outbreaks are frequent in the US, but rare in Europe. This could be due to the different style of fermentation. In European style sausage production the contribution of Gram-positive bacteria (lactic acid bacteria, staphylococci/micrococci) is greater than those in American style procedures (Figs 2, 3). Moreover, it is well known that there is competition and interaction in mixed populations of micro-organisms. These can influence the numbers of *Staph.*

aureus that may be present in meat and meat products stored under different conditions (Minor & Marth 1971; Liepe 1983; Gill 1986; Jones 1989). Indeed, in food technology microbial antagonism can be exploited to suppose the growth of undesirable bacteria (e.g. *Staph. aureus*). This can be achieved by two strategies:

(a) the ability by one organism to use nutrients which others cannot utilize, e.g. the use of gluconate by pseudomonads (Iandolo *et al.* 1965; Nychas *et al.* 1988); and
(b) elimination of competitors by excreted metabolites, antibiotics (e.g. H_2O_2, nisin, lactic acid, diacetyl, micrococcins) which inhibit growth of competing bacteria (Hurst 1973; Jay 1982).

The effect of lactic acid bacteria on the growth of *Staph. aureus* and *Staph. epidermidis* in broth culture and fermented meat has been studied by many workers (Kao & Frazier 1966; Daly *et al.* 1973; Haines & Harman 1973a, b; Niskanen & Nurmi 1976; Smith & Palumbo 1978a; Raccach *et al.* 1979; Bartholomew & Blumer 1980a, b; Metaxopoulos *et al.* 1981a, b; Raccach 1986). The inhibitory effects of starter culture (such as lactic acid bacteria) has been attributed either to lactic acid and hydrogen peroxide (Kandler 1983). A range of lactic acid bacteria has been shown to have an inhibitory effect on growth and enterotoxin production by *Staph. aureus* (Haines & Harman 1973a; Metaxopoulos *et al.* 1981a, b; Raccach 1986). As mentioned above, strains of lactobacilli produce peroxides (Fig. 4). These may not only cause discoloration and rancidity of fermented sausages but may well act as antimicrobial agents. For reasons already discussed the lowering of the oxidation/reduction potential of the environment by lactic acid bacteria could well inhibit the growth of *Staph. aureus* (Barber & Deibel 1972). The growth and enterotoxin production of *Staph. aureus* have been shown to be inhibited by pseudomonads (Troller & Frazier 1963a, b; McCoy & Faber 1966), *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Escherichia coli*, *Bacillus cereus* (Noleto *et al.* 1987), coliforms and *Proteus* spp. (DiGiacinto & Frazier 1966; McCoy & Faber 1966), *Serratia marcescens* (Troller & Frazier 1963b). Yeasts also appear to effect the growth of *Staph. aureus* (Nussinovitch *et al.* 1987.)

6. Acknowledgements

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Chapter 2

Materials and Methods

Section A; Meat stored under different gaseous conditions (Chapter 3).

Experimental design

Beef of normal pH (5.8) as well as higher pH (6.4) pork and beef were studied. In each case approximately 2x5 Kg of minced or whole meat was purchased from a local butcher around 09:00h. It was transported to the laboratory within 30 minutes and held at about 1° C in an ice box for 1-2h. Whole meat was minced with a clean mincer (hot water and detergent). The minced meat was portioned (*ca.* 200-250g) into 48 rigid plastic (Dyno Norway, cat. pac 515 3) containers. Each container was enclosed in a polyethylene bag (oxygen permeability, $P=2.88 \times 10^{-10}$ cm^3 {STP} cm cm^{-2} s^{-1} cm Hg^{-1}) which was evacuated with a vacuum pump and flushed three times before filling with 100% CO_2 or N_2. Some packs contained air. The bags were sealed by heat leaving a gas space of approximately 4l. The sixteen containers used with each atmosphere were stored at 3 ± 0.2° C for *ca.* 2 weeks. On every sampling occasion, two containers from each treatment were removed for analysis. This experiment was done on two occasions.

Microbiological analysis

Samples of minced meat (25g) were weighed out aseptically, 225 ml sterile 1/4 strength Ringer's solution added and homogenized with a stomacher (Lab Blender 400; Seward Medical U.A.C House, London) for 60s

at room temperature. Decimal dilutions in 1/4 strength Ringer's solution were prepared and duplicate portions (1 or 0.1ml) of appropriate dilutions mixed or spread on the media given in Table 2.1 for the isolation of particular groups of microorganisms such as:

a. Total Viable Count (TVC)
b. *Pseudomonas* spp.
c. Enterobacteriaceae
d. *Brochothrix thermosphacta*
e. Yeasts
f. Lactic acid bacteria

As a matter of routine, three randomly selected colonies from every medium were Gram-stained and examined microscopically to check the selectivity of a medium. On some occasions isolates were identified with the API system. Table 2.1 lists the media and incubation periods used in this study.

Physico-chemical and other analyses

Determination of pH. Immediately after microbiological sampling, the pH of the 1 in 10 dilution of a mince sample was measured with a Metrohm 691 pH meter.
The extract release volume (ERV) was determined as described by Jay (1964). Twenty five g of ground meat was reduced to a fine suspension with 100ml water in an Omni mixer (Gallenkamp & Co. Ltd.) for two minutes exactly. The suspension was filtered through a Whatman No 1 filter paper and the volume which passed through in 15 min recorded.

Determination of Low Molecular Weight substances

Sample preparation

Minced meat (25g) was reduced to a fine suspension with 100 ml (3-5° C) cold water in an Omni mixer (Waring,

TABLE 2.1 Media, incubation periods and temperatures used for the enumeration of the microbial flora in minced beef or pork stored under different gaseous conditions at 3°C

Group/organism	Medium*	Incubation period (days)	Incubation temperature (°C)
Total Viable Count	Plate Count Agar (0.1ml;Spread plate)	3	25
Pseudomonas spp. (Mead & Adams 1977)	Pseudomonas Agar Base + CFC supplement (0.1ml;Spread plate)	3	25
Enterobacteriaceae	Violet Red Bile Glucose Agar (1ml;Pour plate)	1	37
Brochothrix thermosphacta (Gardner 1966)	S.T.A.A$ (0.1ml;Spread plate)	2	25
Yeasts	Rose Bengal Chloramphenicol Agar (0.1ml;Spread plate)	5	25
Lactic acid bacteria	Mann, Rogosa Sharp agar (1ml;Pour plate)	4	25

* : Media obtained from Oxoid when available, otherwise made from basic ingredients in the laboratory.
$: Gardner's (1966) medium was supplemented with streptomycin sulfate (500μg ml^{-1}), thallus acetate (50μg ml^{-1}) and actidione 50μg ml^{-1})

New Hartford). The suspension was agitated (orbital shaker, 100 rev/min, 45 min) at 3° C, centrifuged (2000 g; 5 min) and filtered.

The clear filtrate was used to determine the following.

Total sugars by the method of Roe (1954), ammonia by the method of Chavey & Marbach (1962) and soluble proteins according to Lowry *et al.*(1951). Each of these methods included a known amount of the substance sought.

The GOD-Perid kit (Boehringer, Manheim GmBH; enzymatic analysis) was used to determine the concentration of glucose.

Volatile Fatty acids

Sample preparation

Ten ml of the clear filtrate noted above was filtered through a bacteriological filter (0.2µm) and stored at -80° C until analysed.

The volatile fatty acids (VFA) and other volatile compounds in the filtrate were determined by gas chromatography using a Perkin Elmer Gas - Liquid Chromatography Instrument, as follows:

The thawed sample (10 úl) was injected directly into a 2m x 6.25 mm column (10% SP-1000, 1% H_3PO_4 100-120 Mesh Chromosorb W-AW and programmed from 90 to 180° C at 4°C/min with He (40 ml/min) as carrier gas and a Flame Ionization Detector (FID). Peak identification was by comparison of retention times with authentic compounds on the above and on Chromosorb 101 80-100 Mesh.

Lactate and gluconate determination

Sample preparation

To deproteinized sample, a quantity (1-2g) of homogenised minced meat was added to 5ml of ice-cold 1N

perchloric acid, shaken vigorously and centrifuged (15 min, 2000 g). The supernatant was filtered through Whatman No 1 filter paper and the clear filtrate was used to determine lactate by the method of Gutman and Wahlefeld (1974) and gluconate by the method of Mollering and Bergmeyer (1974) with the appropriate controls .

Section B; The Staphylococci of Greek salami (Chapter 4).

Sampling - survey

The survey was done to characterize dry fermented sausages. For this purpose samples were taken from the dispatch department of different companies at the time when products were ready to go to the market. They were brought to the laboratory and analyzed immediately. Some were stored at 4-5° C for the shelf-life test. Samples were taken every week and analyzed microbiologically.

Microbiological analysis

The analysis was done by the methods listed in Table 2.1 as well as in Table 2.2. Isolates from the media (Table 2.2) were identified further by the API system.

Physico-chemical analysis

The a_w and the moisture content were determined as follows:

Water activity was determined with the Thermoconstanter Humidat-TH2 (Novosina, Zurich, Switzerland). The salami samples for analysis were cut into small pieces and filled into the sample bowls provided by the manufacturer. The sample bowls were put into the measuring chamber at a preselected temperature (23° C). The equilibrium humidity was read off the display in % RH when the value remained unchanged for several minutes and the indicated temperature corresponded to the preselected value.

The moisture content was determined as described in AOAC (1984). Sausage meat was removed from casings, passed through a meat chopper with plate openings of 3mm

TABLE 2.2 Media, techniques, incubation periods and temperatures used for the enumeratiuon of specific microorganisms in dry, semi-dry sausage during storage period at 3 °C.

Group/organism	Medium*	Incubation period (days)	Incubation temperature (^{0} C)
Staphylococcus aureus&	Baird-Parker(1)	1	37
Staphylococci&&	Mannitol Salt agar(2)	3	32
Staphylococci#	Schleifer and Kramer (1980)(2)	3	32

1 : Spread technique, 0.1 ml x 2 plates
2 : Pour plate, 1 ml mixed in 15 ml medium followed by 5 ml overlay
* : Media obtained from Oxoid when available, otherwise made from basic ingredients in the laboratory.
& : Black shiny colonies were enumerated
&&: Presumptive coagulase-positive staphylococci separated from coagulase - negative.
: Separation of staphylococci from micrococci.

and mixed thoroughly after grinding. Aluminium dishes of 50mm (diam.) and 30mm (depth) were used for drying. Samples containing *ca* 2 g dry matter were dried in the dishes with lids removed for 16-18 hours at 100-102° C in an air oven (Memmert 854 Schwabach, W.Germany). Dishes were cooled in a desiccator and weighed to constant weight. Moisture was calculated and reported as percentage loss in weight of samples.

Experimental design

The statistical analysis of three and four way analysis of variance was done with SYSTAT software packaging and MINITAB (Penn University, USA).

Section C; Laboratories studies of sausage fermentation (Chapter 5).

Experimental design

Four different inoculation regimes involving four batches of sausages were done. The first batch of sausages (the 'natural' fermentation) were fermented naturally without any deliberate inoculation. Starter cultures of *Lactobacillus plantarum, Staphylococcus carnosus* strain TM-300 and DM-20501 and a mixture of *Lactobacillus plantarum and St. carnosus* TM-300 and DM-20501 were used for some of the batches. The starter cultures were grown in MRS broth (no acetate present) supplemented with 0.2% (w/v) glucose for 24h at 25°C before inoculation of the meat mixture.

The same sausage formulation was used for the four batches. It comprised the following ingredients: lean beef, 47.37 %. lean pork, 47.37 %, sodium nitrite, 0.01 %, potassium nitrate, 0.02, sodium chloride, 2.38 %, sucrose, 0.48 %, glucose, 0.96 % and black pepper 0.18 %.

Sausage preparation

Beef from the flank and pork from the shoulder were purchased from a local butcher and ground through an 8mm plate in a laboratory mincer. Lean beef (237g) and lean pork (237g) were mixed together and stored at -18° C. The meat of each batch of sausage was taken from the stored samples, thawed (at 3-4°C), reground through a 4-mm plate and mixed with curing salts, black pepper, glucose and sucrose for 2 min in a Hobart H-600 mixer. Then the water

and, when appropriate, the starter cultures were added and the mixture was blended for a further 3 min. The initial temperature of the mixtures was approximately 0° C and increased to 5° C during 3 min of blending. Each sausage batch was put into three different types of containers:

1) Beakers(50ml) contained 50g of sausage mix, the surface of which was sealed with melted paraffin wax (melting point, 60°C) in order to maintain anaerobic conditions .

2) Fibrous casings (Cutisin; 41mm diameter) were stuffed by machine with the sausage mix and separated by linking in ca 100g pieces .

3) Polyethylene bag - a 100g of sausage mix was put into the bag which was sealed under vacuum using a Henkovac 1700 sealer (Holland).

Eight (8) experiments were done in total. Two, four and two replicate sausage batches were prepared using types 1,2 and 3 containers respectively. Fermentation took place in an incubator at 23° C with or without air circulation. In each experiment samples were removed on the 1st, 2nd, 3rd, 4th, 6th, 7th, and 9th day of fermentation and analyzed. A sample (25g) was added to 225 ml sterile water and homogenised with a stomacher (Lab Blender 400; Seward Medical U.A.C House, London). Dilutions were prepared in Ringer solutions and appropriate dilutions plated on Plate Count agar, *Pseudomonas* Agar, Mann-Rogosa-Sharpe Agar, Rose-Bengal Chloramphenicol agar, Violet Red Bile Glucose agar and Mannitol Salt Agar (Tables 2.1 and 2.2)

Physicochemical changes were analysed by the methods given in A and B sections above.

Section D; Studies of the factors that affect the growth of *Lactobacillus plantarum* and *Staphylococcus carnosus* as well as their physicochemical attributes in broths (Chapter 6)

Organisms

The following bacteria from the Culture Collection of University of Bath were used alone or in combination; *Lactobacillus plantarum, Staphylococcus carnosus*, strain TM-300 and DM- 20501. These were maintained at 4°C on MRS agar and PCA slopes respectively.

Media

The following media were used to study the growth and physicochemical attributes of the microorganisms:

1. Nutrient broth No 2 (Oxoid) supplemented with yeast extract (Oxoid), 2(%) and thiamine, 0.1(%), pH 6.4.
2. MRS broth without glucose, sodium acetate and triammonium citrate, pH 7.2.
3. NZ amine medium (NZA) containing (g/l): N-Z amine A (Sheffield Chemical Co. Norwich, N.Y , USA), 40.0; yeast extract (Oxoid, L21), 4.0; K_2HPO_4, 1, pH 7.15, and
4. Brain Heart Infusion (BHI) containing (g/l) BHI (Lab M), 17.5; tryptose (Lab M), 10.0; NaCl, 5.0; Na_2HPO_4, 2.5, pH 7.1.

The media were sterilised by autoclaving (121° C for 15 min). In some experiments glucose (20%), sterilised by filtration (0.2 μm; Millipore), was added to give a final concentration of 0.2% (w/v) of medium.

Before each experiment, the bacteria were subcultured as follows: a). Bottles (20ml) containing nutrient broth

No2 were inoculated from a single colony of a 24h old stock culture and incubated at 37° C for 24 h. b). An inoculum of each bacterium from these bottles was added to another bottle of medium and incubated for another 24 h at 37° C, c). Portions (0.2ml) of each culture were used to inoculate duplicate bottles of the medium (75 ml). The cultures were incubated at 37° C with or without shaking (200 rev/min) in an oscillator for 24h. At regular intervals samples (5ml) were removed aseptically from each culture and used immediately to determine pH and microbial growth (as described below) before being frozen at -80° C for other determinations.

Microbial growth

Microbial growth was determined by measuring the optical density at 550 nm with a Carlo-Erba UV/VIS spectrophotometer and/or by viable counts. For the latter, serial 10-fold dilutions in 1/4 Ringer's (Oxoid) solution were prepared for each sample. Bacterial counts were made by the pour plate method (in dublicate) using 1ml of an appropriate dilution and 15ml of MRS followed by 5ml overlay or Baird-Parker(BP) medium, MASA and BP media (Tables 2.1 and 2.2) for the enumeration of *L. plantarum* and *St. carnosus* respectively.

Physico-chemical changes

pH Determination

The pH value was recorded electrometrically (Metrohm 691 pH meter).

Chemical analysis

Sample preparation

Frozen samples of the cultures noted above were

allowed to thaw at room temperature and the cells removed by centrifugation (4000 g for 5 min).

The concentration of glucose in the supernatant fluid was assayed using the GOD-Perid kit (Boehringer,Mannheim GmBH).

Acetate was measured by the acetic acid test kit (Boehringer, Manheim GmBH) and lactate by the method of Gutman and Wahlefeld (1974) without prior deproteinization.

After appropriate dilution of the supernatant fluid, the total soluble proteins were determined by the method of Lowry *et al.*(1951) - Sigma assay Kit; with bovine serum albumin as a standard.

The amount of the high molecular weight proteins in a medium was determined by the method of Sedmak and Grossberg (1977) with bovine serum albumin as a standard.

The volatile compounds produced during the growth of these organisms in a medium were determined with GLC as described in Section A above.

The profile of proteins in a medium

The protein profile of an uninoculated and inoculated growth medium was determined by sodium dodecyl sulphate (SDS)- polyacrylamide gel electrophoresis on gradient gels (PAA 4/30; Pharmacia Fine Chemicals) as described by Reynolds *et al.* (1988). After overnight staining with 0.1% (w/v) Coomassie blue R-250 in 25% (v/v) methanol and 10% (v/v) acetic acid, gels were destained electrophoretically at 24 V for 40 min in 25% (v/v) methanol and 10% (v/v) acetic acid with a GT-4II gel destainer (Pharmacia).

Chapter 3

Microbiology of meat under different gaseous conditions*

Introduction

Meat undergoes progressive deterioration from the time of slaughter until prepared for consumption. Deterioration can be rapid if appropriate storage conditions are not used. In the retail meat trade, chill storage was the first widely used means of delaying bacterial spoilage. Ayres (1960 a,b) demonstrated that off-odours and slime were notable features of red meats stored in a normal atmosphere at chill temperatures. Off-odours were present when the population of pseudomonads were of the order of 10^7 per cm^2 and slime when these organisms occurred at 10^8 per cm^2. In practice off-odours become evident when the pseudomonads have exhausted the glucose and lactate present in meat and begin to metabolise the amino acids (Gill 1976,1982). These observations have been confirmed in many studies (Enfors *et al.* 1979; Nychas and Board 1985) and it is now well established that, under these storage conditions, three species of *Pseudomonas* occur, *Ps. fragi*, *Ps. fluorescens* and *Ps. lundensis* (Molin and Ternstrom 1986).

It is well known also that organisms show the greatest tolerance to a single adverse environmental factor when all other ecological conditions are optimal (International Commission on Microbiological Specification for Foods, CMSF 1980). It was this widely accepted ecological concept that led Leistner and Rodel (1976) to introduce the 'hurdle concept' to food

*The major part of this study was published in International Journal of Food Science and Technology (1990) **25**:389-398.(off-print in Appendix)

microbiology. Indeed, several 'hurdles' or inhibitory factors have been adopted to extend the shelf life of meat. Modified atmosphere packaging (MAP) is the most commonly used method of imposing such hurdles.

The atmosphere may be 'modified' by vacuum packaging or storage of meat in atmospheres containing a mixture of CO_2 and O_2 (Gill and Molin 1991). It needs to be stressed (see Table 1.3, Literature Review) that each of these atmospheres selects a microbial flora dominated by Gram-positive bacteria (principally *Brochothrix thermosphacta* and lactic acid bacteria) rather than the Gram-negative ones that develop on meat stored under chill conditions in a normal atmosphere. As the former are much more slow growing than the latter, the shelf-life of meat is extended. It needs to be stressed also that there are differences in the metabolic attributes of these two groups of spoilage organisms and that these are manifested at different times and in different ways as judged by odours coming from the meat. This topic was recently reviewed by Dainty and Mackey (1992).

Carbon dioxide, nitrogen, oxygen and, for experimental purposes, carbon monoxide have been used in modified atmosphere packs (Clark *et al.* 1976). Nitrogen is chemically inert at normal atmospheric pressure, whilst carbon dioxide affects the growth and composition of the contaminating flora (Enfors and Molin 1978; Blickstad and Molin 1983; Daniels *et al.* 1985; Jones 1989). It has been found that at the end of storage life, *Brochothrix thermosphacta* and lactic acid bacteria were the major contaminants on beef, pork or lamb stored in modified atmospheres of 20-100% of CO_2 - O_2 or N_2 as the additional gases (Newton *et al.* 1977; Erichen and Molin 1981; Nychas and Board 1985). Nitrogen alone was found to have no effect on bacterial growth on meat (Enfors *et al.* 1979).

While the microbiogical changes are well established, the chemical ones accompanying the growth of bacteria on meat during storage are not so well understood (Jay 1986;

Dainty and Mackey 1992). It was suggested that the metabolism of glucose, lactic acid, certain amino acids, nucleotides, urea and sarcoplasmic proteins can all occur during storage (Gill 1976; Jay 1986). The off-odours formed on spoilage of gas-packed meat have been attributed to volatile short-chain fatty acids produced by bacteria from these substrates (Dainty and Hibbard 1983; Dainty *et al.* 1985; Dainty and Mackey 1992). There is little information about the products produced during carbon dioxide and nitrogen storage.

There is pressure on the small food manufacturers in several EC countries to adopt specifications for quality and safety standards. In anticipation of the adoption of "new" technologies in Greece, the objectives of the present study were to measure the extension of shelf-life produced by MA storage of minced beef or pork having normal or high pH, and to confirm previous work relating spoilage to glucose and gluconate metabolism, as well as to changes in other microbial metabolic end products.

Materials and Methods

The materials and methods are given (pp 36-40) in detail in the section A, Materials and Methods.

Results

The meat used in this study was obtained from local butchers. A common practice among Greek butchers needs to be noted. They purchase meat in carcass form and have efficient chill rooms for storage. Even so it is not uncommon to see meat hanging from hooks in the shop at ambient. Beef came from the flank and pork from the shoulder of carcasses. These were minced in the laboratory and 200-250 g samples stored in a normal atmosphere, CO_2 or N_2 at 2-3° C. Changes in microbial contamination and physicochemical properties were monitored. At the outset of this study pH was used to classify the meat as normal or dry firm dark (DFD). Chemical analysis (see Table 3.1) showed that meat of high pH (6.5 and 6.4 for beef and pork respectively) had the characteristically low lactate and glucose contents of DFD meat but an exceptionally high gluconate content. One might suspect, therefore, that the high pH meat may have been caused by unusual storage conditions rather than the physiological status of the animal at slaughter.

Minced beef of low pH stored at 3°C in CO_2 had significantly lower total viable counts (Plate Count Agar) on the 9th day of storage than that stored in air or N_2 (Table 3.2). Indeed samples of beef stored in 100% CO_2 differed from those samples stored in air and nitrogen by about 2 log cycles at this time. The total aerobic count of minced pork of normal or high pH exhibited this pattern also (Table 3.2). These differences were 2.8 and 2.3 log cycles for low pH (5.8) pork and 1.8 and 1.3 log cycles for that of pH 6.4 . The results obtained with the ammonia analysis showed an overall trend of increasing concentration with time, the smallest increase being associated with meat stored in CO_2. The occurrence of putrid off-odours noted with samples packed in air and in nitrogen did not correlate with the ammonia concentrations.

Table 3.1 Changes in the pH in minced beef of low pH (5.5) or high (6.5) pH and pork of low (5.8) or high (6.4) pH during storage at 3° C under different modified atmospheres

Day of Storage	Type of meat	MA Conditions Air	CO_2	N_2
1	Beef	5.5*	5.5	5.5
3	(low pH)	5.5	5.5	5.6
5		5.6	5.5	5.6
9		6.4	5.7	6.5
11		6.2	5.7	6.1
1	Beef	6.5	6.5	6.5
3	(high pH)	6.6	6.3	6.5
5		7.2	6.5	6.5
9		6.9	6.4	7.1
11		6.8	6.6	6.9
1	Pork	5.8	5.8	5.8
2	(low pH)	5.9	5.7	5.9
4		6.1	5.8	5.9
8		6.5	5.9	6.3
10		6.6	6.0	6.6
12		7.0	6.1	6.9
1	Pork	6.4	6.4	6.4
2	(high pH)	6.6	6.4	6.5
5		6.9	6.4	6.5
7		6.9	6.4	6.9
10		7.1	6.7	6.8

* Average of 4 measurements, the coefficient of variation (CV) > 99%

Each sample was analysed in duplicate
1:10 dilution of minced meat in Ringer solution was tested with a pH meter

Table 3.2 Changes in Total Viable Counts (TVC) of naturally contaminated minced beef meat of low pH (5.5) and pork of low (5.8) and high (6.4) pH stored at 3° C under different modified atmospheres

Day of Storage	Type of meat	Log_{10} number of organisms on meat stored in Air	CO_2	N_2
1	Beef	6.6	6.6	6.6
3	(low pH)	7.5	7.3	**8.5**
5		**8.6**	6.8	8.5
9		9.9	7.6	9.9
11		nd	**8.4**	10.5
1	Pork	4.5	4.5	4.5
2	(low pH)	**6.5**	4.8	5.9
4		8.2	5.4	**6.8**
8		9.2	**6.7**	8.8
10		9.8	7.0	9.3
12		nd	8.3	9.9
1	Pork	6.1	6.1	6.1
2	(high pH)	6.4	6.4	6.4
3		7.9	6.9	7.6
5		**8.5**	7.5	**8.2**
7		8.7	7.9	8.4
9		9.2	7.6	8.7
10		9.8	**8.0**	9.3
11		nd	8.6	9.8

Each sample was analysed in duplicate with duplicate plates per replication. There were no differences in excess of log_{10} 0.3 cfu g^{-1} among the samples of the same treatment

Total Viable Count were determined with Plate Count Agar (Oxoid) incubated at 25° for 3 days

Bold figures, spoilage evident by off-odours

The mean values of the pseudomonad counts on CFC agar are given in Table 3.3 for low pH beef, low pH pork and high pH pork. As it can be seen, storage in 100% CO_2 had a major influence on these counts in all types of meat. After 9 days of storage, samples stored in CO_2 had significantly lower pseudomonad counts than those stored in air or nitrogen. Nitrogen in other words did not have any inhibitory action on the growth of pseudomonads.

The counts of lactic acid bacteria are given in Table 3.4. Large increases occurred in their numbers on all three types of meat during storage in air, carbon dioxide or nitrogen. Indeed, there was no appreciable differences in the extent of growth of lactobacilli between the three test atmospheres during the 10-11 days of storage of the three types of meat (Table 3.4). Moreover the growth of lactobacilli did not cause an acid drift in any of the samples (Table 3.1).

Growth of *Br. thermosphacta* in low-pH beef, low-pH pork and high-pH pork was unaffected by pH during storage under nitrogen or aerobic conditions. Carbon dioxide slowed the growth rate of *Br. thermosphacta* (Table 3.5) on pork such that the counts of this organism in CO_2 storage were approximately 1 log lower than those in air or nitrogen storage. Again the initial pH of a sample did not appear to be reflected in either the rate or extent of growth of this organism.

The initial yeast counts on low-pH beef and high-pH pork were $\log_{10}$ 3.4 and 4.8 g^{-1} respectively. After 9 days of storage under air or nitrogen, counts had reached $\log_{10}$6.5 and 6.2 for beef and $\log_{10}$ 7 g^{-1} for pork (Table 3.6). It is noteworthy that CO_2 reduced appreciably the growth rate of yeasts on both types of meat regardless of initial pH.

The type and pH of meat did not affect the growth of enterobacteria during storage under aerobic conditions or nitrogen. After 7-9 days of storage, the counts had reached $\log_{10}$ 7 g^{-1} or more (Table 3.7). Carbon dioxide

Table 3.3 Changes in pseudomonad counts of naturally contaminated minced beef meat of low pH (5.5) and pork of low (5.8) and high (6.4) pH stored at 3° C under different modified atmospheres

Day of Storage	Type of meat	Log_{10} number of organisms on meat stored in Air	CO_2	N_2
1	Beef	5.4	5.4	5.4
3	(low pH)	6.9	5.9	7.2
5		8.1	6.4	8.2
9		9.6	7.5	9.6
11		nd	7.4	10.2
1	Pork	3.7	3.7	3.7
2	(low pH)	4.2	3.9	4.9
4		6.7	4.1	5.8
8		7.7	4.5	7.8
10		9.1	5.3	8.5
12		nd	6.2	9.7
1	Pork	6.5	6.5	6.5
2	(high pH)	7.5	6.0	7.3
3		8.4	7.0	8.1
5		8.5	7.1	8.2
7		8.6	7.2	7.9
9		9.4	7.8	8.9
10		nd	7.4	9.0
11		nd	8.4	9.5

Each sample was analysed in duplicate with dublicate plates per replication. There were no differences in excess of log_{10} 0.3 cfu g^{-1} among the samples of the same treatment

The pseudomonads were isolated on CFC Agar (Oxoid) incubated at 25° C for 3 days. The selection of the medium was checked with Gram staining of 3 randomly selected colonies from each plate having a countable dilution. In addition the isolates were purified and tested with the API system. The following species were identified ;*Ps. capacia*,*Agrobacter radiobacter*, *Flavobacterium multivorum*, *Past. pneumotropica* *Ps. fluorescens*

nd : no determined

Table 3.4 Changes in lactobacilli counts in naturally contaminated minced beef of low pH (5.5) and pork of low (5.8) and high (6.4) pH stored at 3° C under different modified atmospheres

Day of Storage	Type of meat	Log_{10} number of organisms on meat stored in Air	CO_2	N_2
1	Beef	3.5	3.5	3.5
3	(low pH)	3.8	3.9	4.4
5		4.8	4.8	5.4
9		6.2	6.5	6.7
11		nd	7.2	7.6
1	Pork	3.4	3.4	3.4
2	(low pH)	4.0	4.4	5.2
4		6.2	5.4	6.2
8		7.7	6.7	7.7
10		8.2	7.8	8.4
12		nd	8.2	9.2
1	Pork	4.4	4.4	4.4
2	(high pH)	5.4	5.4	5.4
3		5.1	5.2	4.9
5		7.1	5.7	6.9
7		6.4	6.0	6.4
9		6.9	7.1	6.3
10		7.7	6.4	7.8
11		nd	7.4	8.0

Each sample was analysed in duplicate with duplicate plates per replication. There were no differences in excess of log_{10} 0.3 cfu g^{-1} among the samples of the same treatment

Lactobacilli were isolated on MRS Agar (Oxoid) incubated at 25° C for 4 days. The selection of the medium was checked with Gram staining of 3 randomly selected colonies from each plate having a countable dilution. No organisms were identified

nd no determined

Table 3.5 Changes in counts of Brochothrix thermosphacta in naturally contaminated minced beef of low (5.5) pH and pork of low (5.8) or high (6.4) pH stored at 3° C under different modified atmospheres

Day of Storage	Type of meat	Log_{10} number of organisms on meat stored in Air	CO_2	N_2
1	Beef	4.5	4.5	4.5
3	(low pH)	5.9	5.4	6.9
5		7.9	6.0	7.7
9		8.9	8.5	8.9
11		nd	8.7	10.0
1	Pork	3.3	3.3	3.3
2	(low pH)	4.0	4.2	5.2
4		5.3	5.2	5.8
8		7.2	6.5	7.5
10		8.8	7.3	8.2
12		nd	8.2	9.4
1	Pork	5.1	5.1	5.1
2	(high pH)	6.0	5.6	6.0
3		6.2	5.6	6.9
5		7.7	6.6	7.2
7		7.9	7.4	8.1
9		8.3	7.1	7.8
10		8.7	7.8	9.0
11		nd	8.5	9.2

Each sample was analysed in duplicate with duplicate plates per replication. There were no differences in excess of log_{10} 0.3 cfu g^{-1} among the samples of the same treatment

The organism was isolated on STAA Agar (Gardner, 1966) incubated at 25° for 2 days. The selection of the medium was checked with Gram staining of 3 randomly selected colonies from each plate having countable dilution. Microscopic examination allowed identification of this organism - long chain of Gram positive cells with a few Gram positive coccobacilli in chains or clumps

nd no determined

Table 3.6 Changes in yeast counts in naturally contaminated minced beef of low (5.5) pH and pork of low (5.8) and high (6.4) pH stored at 3° C under different modified atmospheres

Day of Storage	Type of meat	Log_{10} number of organisms on meat stored in Air	CO_2	N_2
1	Beef	3.4	3.4	3.4
3	(low pH)	3.9	3.4	4.9
5		4.9	3.9	4.7
9		6.5	4.8	6.2
11		nd	5.7	7.3
1	Pork	4.8	4.8	4.8
2	(high pH)	nd	nd	nd
3		5.6	4.7	5.3
5		6.7	4.7	6.6
7		nd	nd	nd
9		7.0	4.8	7.0
10		nd	nd	nd
11		nd	5.2	7.3

Each sample was analysed in duplicate with duplicate plates per replication. There were no differences in excess of log_{10} 0.3 cfu g^{-1} among the samples of the same treatment

The yeasts were isolated on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° C for 5 days

nd no determined

Table 3.7 Changes in Enterobacteriaceae counts in naturally contaminated minced beef of low (5.5) pH and pork of low (5.8) and high (6.4) pH stored at 3° C under different modified atmospheres

Day of Storage	Type of meat	Log_{10} number of organisms on meat stored in Air	CO_2	N_2
1	Beef	2.9	2.9	2.9
3	(low pH)	4.4	3.5	5.5
5		6.2	4.2	5.4
9		7.7	4.7	6.9
11		nd	5.0	7.5
1	Pork	2.2	2.2	2.2
2	(low pH)	4.0	2.2	4.2
4		5.7	2.7	5.2
8		6.5	3.6	6.2
10		nd	4.6	7.5
12		nd	5.1	7.8
1	Pork	2.8	2.8	2.8
3	(high pH)	4.2	3.7	4.7
5		6.1	3.7	5.8
9		6.8	4.2	6.5
11		nd	4.9	6.7

Each sample was analysed in duplicate with duplicate plates per replication. There were no differences in excess of log_{10} 0.3 cfu g^{-1} among the samples of the same treatment

Enterobacteriacae were isolated on VRBG Agar (Oxoid) incubated at 37° for 24 hours. The selection of the medium was checked with Gram staining of 3 randomly selected colonies from each plate having countable dilutions. In addition 32 isolates were purified and tested with the API system. The following species were identified: *Enterobacter cloacae,Klebsilla oxytoca, Enterobacter aerogenes, Enterobacter sakazakii, Citrobacter freundii, E.coli,Salmonella arizona,Serratia* spp.

nd no determined

reduced the rate of growth of enterobacteria such that by the 11th day of storage the log counts were about 2 log units lower than those on meat stored in air or nitrogen.

The results presented in Tables 3.2 - 3.7 are in agreement with those published by many other workers (Newton *et al.* 1977; Erichsen and Molin 1981; Blickstad and Molin 1983). If a difference of 2 $\log_{10}$ cycles in the counts of TVC is taken as evidence of preservation, then it is evident that CO_2 was an effective preservative system. Of course storage in CO_2 caused the development of a brown colour in the meat.

Physicochemical analysis

A progressive increase of pH values was observed throughout storage of all meat samples and, as noted above, the growth of lactobacilli did not appear to influence to any great extent the alkaline drift in stored minced meat. In a CO_2 atmosphere the pH increase of normal and high pH beef and pork was small, 0.1 and 0.3 units respectively (Table 3.1). In the case of storage in air or nitrogen, the increase in pH was greater, 0.6 to 1.2 units. The highest increases occurred in the meats of low pH.

The concentrations of glucose, total sugars and lactate were higher on the first day in normal pH than in beef or pork of high pH (Table 3.8, 3.9 and 3.10). In all cases the loss of glucose occurred later during storage of normal pH beef and pork under carbon dioxide than in air or nitrogen. This trend was not as pronounced in beef and pork of high pH, both of which contained much less glucose. The patterns in the changes in the concentration of total sugars were similar to those discussed above, as can be seen in Table 3.9. Despite progressive increases in the lactobacilli counts, the lactic acid content decreased in all samples of normal and high pH beef as well as pork of high pH (Table 3.10). Even so after 9 days of storage, however, the amount of

Table 3.8 Changes in glucose content in minced beef of low (5.5) pH and pork of low (5.8) or high (6.4) pH during storage at 3° C under different modified atmospheres

Day of Storage	Type of meat	Glucose (mg $100g^{-1}$) MA Conditions Air	CO_2	N_2
1	Beef	111*	111	111
3	(low pH)	97	91	58
5		91	91	30
9		29	81	22
11		13	61	36
1	Beef	65	65	65
3	(high pH)	34	57	35
5		26	44	31
9		15	30	35
11		10	17	20
1	Pork	110	110	110
2	(low pH)	85	120	122
4		45	100	86
8		25	90	56
10		10	85	34
12		0	45	7
1	Pork	60	60	60
2	(high pH)	32	62	45
5		28	70	51
7		16	40	38
10		8	24	22

* Average of 4 measurements, the coefficient of variation (CV) >95%

Each sample was analysed in duplicate with the GOD-Perid kit (Boehringer, Manhein GmBH;enzymatic analysis)

Table 3.9 Changes in total sugars content in minced beef of low (5.5) or high (6.5) pH and pork of low (5.8) or high (6.4) pH during storage at 3° C under different modified atmospheres

Day of Storage	Type of meat	Total Sugars (mg $100g^{-1}$) MA Conditions Air	CO_2	N_2
1	Beef	150*	150	150
3	(low pH)	120	140	130
5		110	145	100
9		70	150	100
11		50	160	40
1	Beef	110	110	110
3	(high pH)	80	90	100
5		80	95	75
9		72	85	60
11		42	60	35
1	Pork	122	122	122
2	(high pH)	86	110	100
5		80	105	67
7		62	95	55
10		33	55	38

* Average of 4 measurements, the coefficient of variation (CV) > 95%

Each sample was analysed in duplicate by the method of Roe (1954)

Table 3.10 Changes in lactate content in minced beef of low (5.5) or high (6.5) pH and pork of low (5.8) or high (6.4) pH during storage at 3° C under different modified atmospheres

Day of Storage	Type of meat	Lactate (mg $100g^{-1}$) MA Conditions Air	CO_2	N_2
1	Beef	380*	380	380
3	(low pH)	304	361	375
5		241	300	305
9		117	252	197
11		87	317	201
1	Beef	160	160	160
3	(high pH)	132	170	144
5		110	132	152
9		84	136	78
11		71	100	92
1	Pork	260	260	260
2	(low pH)	230	270	270
4		190	260	230
8		160	230	200
10		110	225	140
12		70	230	95
1	Pork	220	220	220
2	(high pH)	200	230	170
5		180	210	190
7		100	190	123
10		90	170	143

* Average of 4 measurements, the coeffincient of variation (CV) > 95%

Each sample was analysed in duplicate by the method of Gutman and Wahlefeld (1974)

lactic acid remaining in meat stored in CO_2 was always greater than in that stored in nitrogen or air. The diminution of lactic acid content in all minced meats was paralleled by an increase in pH values (Table 3.1).

The gluconate content in both beef and pork of high pH was higher than that in beef and pork of normal pH on the first day of storage (Table 3.11). Changes in gluconate content in normal pH beef were small regardless of the storage atmosphere, but the high initial levels of gluconate in beef and pork of high pH were lost rapidly with meat stored in air, but more slowly in N_2. The loss was markedly delayed with CO_2 storage. In the case of normal pH pork, gluconate was lost almost completely after 12 days of storage in air or nitrogen. With pork of low or high pH, there was a gradual increase of gluconate up to the 10th day of storage in CO_2. On the 10th day of storage of pork of low pH the level of gluconate was higher than the initial value and above the level of gluconate in pork of high pH.

Extract release volumes (ERV) tended to decrease in all samples other than beef of low pH under CO_2 (Table 3.12). Water soluble proteins and ammonia gradually increased in concentration in all samples as the storage time progressed up to 9 days (Table 3.13 and 3.14).

Ethanol, diacetyl, acetic acid and an as yet unidentified compound (Table 3.15 and 3.16) were always present in minced meat sampled throughout storage under the different gaseous environments. There was no evidence that the presence of any of these were related to the development of off-odours. Acetoin, propionic, *iso*-butyric, *iso*-valeric, valeric, *iso*-caproic and caproic acids were detected (gas chromatography) and associated with the off-odours of minced beef or pork. The unknown compound was the dominant peak at the end of storage of beef of low or high pH or pork of high pH stored in carbon dioxide and in beef of low pH stored in nitrogen.

Table 3.11 Changes in gluconate content in minced beef meat of low (5.5) or high (6.5) pH and pork of low (5.8) or high (6.4) pH during storage at 3° C under different modified atmospheres

Day of Storage	Type of meat	Gluconate ($\mu g\ g^{-1}$) MAP Conditions Air	CO_2	N_2
1	Beef	19*	19	19
3	(low pH)	23	20	53
5		22	12	21
9		16	25	14
11		12	16	12
1	Beef	94	94	94
3	(high pH)	11	95	70
5		4	85	41
9		7	132	6
11		4	30	5
1	Pork	45	45	45
2	(low pH)	90	52	60
4		45	60	70
8		15	118	80
10		0	156	44
12		0	65	9
1	Pork	95	95	95
2	(high pH)	102	89	124
5		64	153	52
7		34	136	63
10		12	90	65

* Average of 4 measurements, the coefficient of variation (CV) > 95%

Each sample was analysed in duplicate by the enzymatic method of Mollering and Bergmeyer (1974)

Table 3.12 Changes in extract release volume (ERV) in minced beef of low (5.5) or high (6.5) pH during storage at 3° C under different modified atmospheres

Day of Storage	Type of meat	ERV (ml) MA Conditions Air	CO_2	N_2
1	Beef	82*	82	82
3	(low pH)	78	79	60
5		77	76	60
9		50	55	42
11		15	85	45
1	Beef	64	64	64
3	(high pH)	35	42	34
5		22	64	66
9		22	43	29
11		14	20	20

* Average of 4 measurements, the coeffincient of variation (CV) > 95%

Each sample was analysed in duplicate by the method of Jay (1964)

Table 3.13 Changes of soluble proteins content in minced beef of low (5.5) or high (6.5) pH during storage at 3° C under different modified atmospheres

Day of Storage	Type of meat	Soluble Proteins (mg $100g^{-1}$) MA Conditions Air	CO_2	N_2
1	Beef	107*	107	107
3	(low pH)	87	140	143
5		123	145	154
9		154	123	156
11		112	35	132
1	Beef	5	5	5
3	(high pH)	10	6	8
5		65	3	87
9		98	6	126
11		154	63	187

* Average of 4 measurements, the coefficient of variation (CV) > 95%

Each sample was analysed in duplicate by the Sigma assay kit (Lowry *et al.* 1951)

Table 3.14 Changes in ammonia content in beef of low (5.5) and high (6.5) pH during storage at 3° C under different modified atmospheres

Day of Storage	Type of meat	Ammonia (A 625 nm) MAP Conditions Air	CO_2	N_2
1	Beef	0.08*	0.08	0.08
3	(low pH)	0.11	0.08	0.10
5		0.13	0.11	0.12
9		0.25	0.11	0.14
11		0.17	0.13	0.16
1	Beef	0.10	0.10	0.10
3	(high pH)	0.10	0.10	0.10
5		0.12	0.12	0.13
9		0.14	0.12	0.16
11		0.17	0.14	0.20

* Average of 4 measurements, the coefficient of variation (CV) > 95%

Each sample was analysed in duplicate by the method of Chavey and Marbach (1962)

Table 3.15 Volatile compounds produced by the naturally contaminated minced beef with low (5.5) and high (6.5) pH stored at 3° C under different modified atmospheres conditions

Packaging	Storage Days	Ethanol	Diacetyl	Unknown	Acetic acid
Normal pH					
	1	28.0	0.1	10.6	61.0
Air	3	16.0	0.9	52.2	30.0
	5	2.3	0.5	81.2	16.0
	9	2.3	0.1	65.5	32.0
	11	49.7	7.2	15.2	27.0
CO_2	3	20.3	0.8	36.8	42.0
	5	1.9	3.8	80.0	14.0
100%	9	1.2	0.3	96.4	2.1
	11	2.0	0.1	92.5	5.2
	3	6.7	0.5	50.2	42.0
	5	2.0	0.2	89.6	8.0
N_2	9	1.5	0.2	86.0	12.6
	11	1.9	0.2	91.7	6.2
High pH					
	1	7.0	1.0	29.0	63.0
	3	5.9	0.7	45.0	48.0
Air	5	6.5	7.4	23.0	63.0
	9	9.5	2.7	40.3	47.5
	11	11.7	1.1	42.3	45.2
	3	12.3	0.9	30.5	56.3
CO_2	5	6.8	2.5	57.5	33.3
	9	3.7	0.4	86.0	10.0
	11	5.2	0.3	91.0	3.0
	3	9.1	0.7	64.3	25.4
N_2	5	5.5	0.6	69.0	25.0
	9	12.0	2.1	54.0	32.0
	11	1.9	0.2	64.0	32.0

Values are the mean of three (3) replicates from each sample, coefficient of variation (CV) > 99%. These are expressed as percentage of the total which derived from the sum of their ereas.
These areas were given by the intergator (Perkin-Elmer 10B) which was linked with the 3B Perkin-Elmer gas chromatograph

Table 3.16 Volatile compounds produced by the naturally contaminated minced pork with high (6.4) pH stored at 3° C under different modified atmospheres

Packaging	Storage Days	Ethanol	Diacetyl	Unknown	Acetic acid
	1	3	2	35	60
Air	3	3	0.5	35	61.5
	6	10	2	40	48
	8	8	1.5	45	45.5
	11	nd	nd	nd	nd
CO_2	3	5	1	45	49
	6	7	0.5	55	37.5
100%	8	6	1	60	33
	11	4	2	80	14
	3	10	2	42	46
	6	8	1	51	40
N_2	8	6	1	59	34
	11	3	0.1	65	32

Values are the mean of three (3) replicates from each sample, coefficient of variation (CV) > 99%. These values are expressed as percentage of the total which derived from the sum of their areas

These areas were given by the intergator (Perkin-Elmer 10B) which was linked with the 3B Perkin-Elmer gas chromatograph

Discussion

The antimicrobial effect of increased partial pressures of CO_2 has been known for a long time. It was evident in this study also. Living cells require CO_2 but if the concentration increases above a certain critical level metabolic activity will be retarded (Gill and Molin 1991).

Theories advanced for the inhibitory effect of CO_2 on aerobic spoilage bacteria include: a) alteration of intracellular pH and consequent effect on intracellular enzyme activities and substrate transport (Wolfe 1980); b) inhibition of decarboxylating enzymes by mass action effect (King and Nagel 1975); c) dissolution of CO_2 in cell membranes with consequent expansion and physical disruption of and loss of membrane functions (Sears and Eisenberg 1961; Enfors and Molin 1981), and d). inhibition of non-decarboxylating enzymes by action of non-polar sites (Ransom *et al.* 1960).

The rate of bacterial multiplication decreases, and the length of the lag phase increases, with increasing levels of CO_2 especially at chill temperatures (Tomkins 1932; Haines 1933). Gram-positive species are more resistant to the effects of CO_2 than Gram-negative ones (Sutherland *et al.* 1977; Siliker and Wolfe 1980; Stier *et al.* 1981). This was evident in this study also. These effects vary with the concentration of CO_2, incubation temperature and water activity of the medium (Wodzinski and Frazier 1961). The increased inhibitory action of CO_2 at lower temperatures is considered to be due to increased CO_2 solubility, as opposed to increased cell susceptibility. The partial pressures used in determining the degree of inhibition by CO_2 on the growth of the food-spoilage bacteria were between 0 and 1 atm (Dixon and Kell 1989). These high partial pressures of CO_2 have a considerable effect in prolonging the shelf-life of unsterilized foods such as meat by virtue of the fact

that they select *Brochothrix thermosphacta* and lactic acid bacteria (Shaw and Nicol 1969; Roth and Clark 1975), and by reducing the growth rate of these organisms (Blickstad *et al.* 1981; Johnson *et al.* 1982; Blickstad and Molin 1983). This was a feature in this study also. Thus the preservative action of CO_2 is not so much due to the control of the total microbial population as to restriction of growth of those types of organisms which have the potential to cause most rapid deterioration. The selection of *Lactobacillus* spp. is considered by some to have an added benefit as many lactic acid bacteria are known to exert an antagonistic effect against other bacteria (Price and Lee 1970; Schroder *et al.* 1980).

The inhibitory effect of CO_2 on Gram-negative aerobic psychotrophic meat bacteria is now well established (Rousett and Renerre 1991; Christopher *et al.* 1980a, 1980b ; Seideman *et al.* 1979; Newton *et al.* 1977; Huffman *et al.* 1975; Ledward *et al.* 1971; King and Nagel 1967 ; Baran *et al.* 1969,1970; Clark and Lentz 1969; Shaw and Nicol 1969; Kraft and Ayres 1952). It was exemplified by the results obtained in this study (Table 3.2).

The role of N_2 on the microflora of meat is still unclear (Christopher *et al.* 1980). Huffman (1974) stated that aerobic plate counts of pork chops stored in N_2 were similar to those of pork chops stored in air. It was confirmed by Huffman (1974) also that high CO_2 atmospheres reduced significantly microbial growth on minced pork stored at 3° C. The inhibition of microbial growth was not the result of lowered amounts of O_2 present in the storage atmosphere, since minced pork stored in N_2 had counts similar to those stored in air. In contrast, Newton *et al.* (1977) reported that microbial counts on lamb chops stored in N_2 were much lower than those stored in air, 80% O_2+20% CO_2, or in 80% air+20% CO_2. Our results agree with Huffman's findings. Possible explanations for the lack of agreement could be differences in the nature of the samples, plating media, conditions of incubation,

sampling methods and composition of the gases (Christopher *et al.* 1980b), especially the composition of N_2. A very low O_2 concentration could cause growth of TVC and pseudomonads (Table 3.2 and Table 3.3). In our experiments the N_2 purity was not verified.

A shift from an initial flora containing mainly Gram-negative aerobic organisms to a predominantly Gram-positive facultative anaerobic microflora dominated by *Lactobacillus* spp. usually occurs in muscle foods during MAP storage. This was the case in the present study. This is beneficial in the sense that the by-products of *Brochothrix thermosphacta* and lactobacilli metabolism are usually produced very slowly and are relatively inoffensive compared to the typical spoilage odours produced by pseudomonads for example. Additionaly of course the unusual Greek practise of storing meat in the shop rather than chill room during shop-hours may be involved also.

Organisms which appear to be a problem in high pH (pH>6) dark, firm, dry meats are *Shewanella putrefaciens*, *Serratia liquefaciens*, and *Yersinia enterocolitica*. All would be inhibited by a typical MAP environment used for fresh meats of low pH (Farber 1991).

In the present work when minced beef or pork (normal and high pH) was stored in CO_2 at 3° C, the shelf-life, - defined as a 100-fold increase in TVC - was almost doubled. In practice nitrogen did not retard microbial growth on beef or pork. This was shown by Enfors *et al.* (1979) also. The growth of Gram-negative bacteria and yeasts was inhibited and *Br. thermosphacta* and lactic acid bacteria became dominant with CO_2 storage. These results agree with those previously reported for bacteria on beef, lamb and pork (Newton *et al.* 1977; Erichsen and Molin 1981; Blickstad and Molin 1983). Our studies do add new data about the fate of meat yeasts, organisms that are not commonly sought in studies of this type. These organisms tended to be inhibited by CO_2. The potential

of the climate of the areas where cattle are raised to influence the shelf-life during chill storage of meat produced thereon was cited in the Literature Review. When our results are compared with those of Nychas *et al.* (1991a), there was no evidence of this feature operating in Greek minced meat.

As carbon dioxide selects a microbial flora on minced meat different from that stored in air or nitrogen, some differences in physicochemical properties might be expected. Jay (1964) and Shelef and Jay (1970) introduced Titrimetric Acidity and Extract Release Volume (ERV), while Sutherland *et al.* (1975) used pH alone for the prediction of bacterial spoilage of fresh beef. Our results with normal beef stored in air (Tables 3.14) agree with those of Jay (1964) and Nychas *et al.*(1991a), in that changes in ERV decreased with microbial growth. In contrast, ERV did not change with carbon dioxide storage. Carbon dioxide, and to a lesser extent nitrogen, influenced pH without appreciable changes in ERV. Similar results have been reported for vacuum-packaged beef (Sutherland *et al.* 1975). The failure of these tests as indicators of spoilage in minced meat in carbon dioxide is probably due both to its effects on the composition of the flora as well as its property as a weak acid (Jones 1989).

As was noted at the outset of the Results section, pH appeared to be an inappropriate means of classifying the meat as normal (pH : 5.5, 5.8 : beef, pork) or dry firm dark (pH : 6.5, 6.4; beef, pork). Although these pH values were associated with characteristically low levels of glucose and lactic acids, the gluconate levels appeared to be unusually high. Indeed these levels may well indicate that storage conditions of the meat prior to mincing had caused a loss of lactic acid and glucose as well as build up in gluconate in the meat having high pH. It was notable that high pH pork had the largest microbial counts of pseudomonads at the beginning of the experiment. These organisms have been associated with

gluconate production (Mitchell and Dawes 1982; Nychas *et al.* 1988). Thus, although the role of glucose in the microbiology of 'normal' meat is well established - off-odours are not produced until glucose and lactate are ex hausted (Gill 1976; Nychas *et al.* 1988), the results presented in this section may be exceptional. The conversion of glucose to gluconate - a characteristic feature in meat exposed to a normal atmosphere during chill storage (Nychas *et al.*1988) - *via* the extracellular glucose - dehydrogenase of pseudomanads provides them with a competitive advantage because gluconate is not so readily utilized by other members of the meat microflora (Farber and Idziak, 1982; Nychas *et al.* 1988). The delay in the utilization of glucose and total sugars, as well as the decrease of gluconate during the early stages of storage of both types of minced meat stored in carbon dioxide, was probably due to high pCO_2 or low pO_2 inhibiting the activity of glucose-dehydrogenase of pseudomonads (Mitchell and Dawes 1982; Nychas *et al.* 1988). The subsequent increase in the concentration of gluconate (Table 3.11) was associated with an increase in the size of the population of pseudomonads (Table 3.3). It would appear therefore that, during storage, the composition of the gas phase in the bags may have changed, possibly due to the diffusion of O_2. The limited changes in gluconate concentration in CO_2 stored meat contrast with those occurring in meat stored in either a normal atmosphere or nitrogen. In both of the latter instances the concentration of glucose and total sugars diminished rapidly, and the transient peak in gluconate concentration occurred at an early stage of storage (Tables 3.8, 3.9, and 3.11).

Gluconate is not connected directly with the production of off-odours, which are mainly caused by sulphur compounds, diacetyl, lactic acid, acetic acid and to, a lesser extent, short-chain branched fatty acids (eg. *iso*-butyric,*iso*-valeric) and to alcohols (e.g. 2,3 butanediol, 2-methylpropanol) (Dainty and Hoffman, 1983;

Dainty *et al.* 1985; Dainty and Mackey, 1992). The putrid off-odour, which was evident in minced meat stored in air, coincided with the increase of ammonia and soluble proteins together with glucose exhaustion (Tables 3.8, 3.12 and 3.13; on the nineth day in both experiments), and the results are in accord with the findings of Gill (1976) and Gill and Newton (1977). As CO_2 inhibited growth - and presumably glucose assimilation by pseudomonads - the dairy/cheesy odours mainly found in samples stored in CO_2 and to a lesser extent in N_2 were no doubt produced by *Br. thermosphacta* and lactic acid bacteria both of which can produce diacetyl and alcohols (Dainty and Hibbard, 1983; Dainty and Hoffman, 1983) exclusively from glucose under aerobic conditions or low O_2 tension (Blickstad and Molin 1984). All of these components were detected by gas chromatography in this study also. Therefore spoilage of stored minced meat is not, as might be inferred from the findings of Gill (1976, 1986) and Gill and Newton (1977), exclusively associated with the onset of amino acid metabolism subsequent to glucose and gluconate depletion.

Although the presence of off-odours is the most rapid and universally accepted indicator of the end of shelf-life of meat stored in air, problems may arise with novel forms of gas packaging. This study has shown that the important changes in microbial metabolites, and their possible role as indicators of incipient spoilage, are determined by storage methods. Indeed, this study demonstrated clearly that each different type of storage condition (air, CO_2, nitrogen) provided unique environments for the growth of particular groups of bacteria.

Chapter 4

The staphylococci of Greek salami

Introduction

The microbiology of fermented sausage is considered in the Literature Review (pp 5-35). It is obvious from this review that little attention has been given to sausages made in Greece.

Bloukas and Paneras (1985) studied the feasibility of producing salami sausages containing reduced amounts of nitrate and nitrite and with or without the addition of potassium sorbate. They directed attention at the organoleptic quality rather than the bacteriological attributes of their products. They stated that nitrate and nitrite can be reduced from 1500ppm to 500ppm and from 200ppm to 100ppm respectively without impairing the quality of salami. In contrast, 1000ppm of potassium sorbate could not substitute for nitrate or nitrite in the development of colour and organoleptic characteristics and stability. These results were to be anticipated in view of our knowledge about the role of nitrate/nitrite in colour development and stability (see p. 31 in the Literature Review). Paneras and Bloukas (1984) carried out a study concerning the physicochemical characteristics of some salami sausages. The values they obtained fell within limits accepted in Greece. Again, however, these workers did not concern themselves with the microbiology of the samples. The bacteriological quality of the dry sausage, "Trikala", and fresh raw (non-ripened) sausages, manufactured in local establishments in the area of Thessalia was investigated by Grigoriadis *et al.* (1988). Mean values of $9x10^{6}g^{-1}$, $8.4x10g^{-1}$ and $3.6x10^{3}g^{-1}$ for aerobic plate counts, coliforms and enterococci counts respectively were

reported. The mean pH value was 5.65±0.45 and a_w was 0.87±0.04. It needs to be stressed that none of the above studies has considered in detail the organisms involved in fermentation.

It has been assumed, of course, that *Lactobacillus* spp. were the most important organisms in this respect. This has been confirmed in a recent study (Samelis, J. pers. comm.). This worker has used both traditional biochemical methods to characterize *Lactobacillus* spp. as well as those based on I6S ribosome analysis. It would appear from preliminary analysis that a *Lactobacillus* sp. other than *plantarum*, the common lactobacillus of fermented sausages worldwide, may be dominant in Greek sausages. As yet there has been no reference at all to the possible occurence of *Staphylococcus carnosus* in Greek sausages produced by fermentation. In the present study attention was given to *Staphylococcus* spp. because, as was evident in the Literature Review, it is now recognised that members of this genus, particularly *St. carnosus*, can be important organisms in the initial fermentation.

Materials and Methods

The methods used in this Section are described in Material and Methods (pp 41-43). Products taken directly from the factories of 7 companies as well as from shops were analyzed.

Results

A preliminary survey was done in order to characterise some physicochemical and microbiological attributes of salami (dry sausage) manufactured by the 7 companies that command the market for this product in the metropolitan area of Athens. Samples were taken from the dispatch

department of the companies. Additionally, salamis of these and other companies were purchased from shops in the Athens' area. Elsewhere in Greece, dry sausages of the salami type are sold under names that do not refer to this generic type of sausage. It was for this reason that samples of the sausage Leukadas were included in the study. The latter samples were obtained from butchers' shops on the island of Leukada. A total of 60 samples of sausages were analysed. In all instances, samples were analysed within 24 hours of purchase.

It is evident from the results summarised in Table 4.1 that the pH values of the sausages fell in the range 4.33-5.7 (mean 4.92, standard deviation 0.45). As an a_w meter was not available throughout the entire study period, only a few results are presented. These do show however a marked range in values, 0.61-0.91 (mean 0.78, standard deviation 0.09). The effect of storage on this attribute is considered below. As the Total Viable Count is used in Greece as a general index of the microbiological quality of sausages, it was included in this study also. It can be seen in Table 4.1 that the total viable counts ranged from $\log_{10}$ 4.06-7.51 c.f.u. g^{-1} (mean 6.05, st. dev. 1.09). In practice there was a relatively low correlation (r=0.698) between the pH of a sausage and the T.V.C.

Lactic acid bacteria isolated on MRS agar were the numerically dominant organisms in the majority of sausage samples with a mean count of $\log_{10}$ 6.18 (range 5.15-7.85, st. dev. 0.91). There was, however, a poor correlation (r=0.547) between the lactobacilli count and the pH of sausages.

As there is no concensus in the literature on the preferred medium for isolating staphylococci from fermented sausages, three were used in this study. It is evident from Table 4.1 that the medium of Schleifer and Kramer (1980) gave the highest counts in the survey

Table 4.1 Physicochemical and microbiological characteristics of 60 samples of dry fermented Greek sausages (salami) obtained from factories and shops[1]

Type of Sausage	pH	Aw	Log_{10} cfu g^{-1} Total Viable Counts (1)	Yeasts (2)	Lactobacilli (3)	Staphylococci (4)	(5)	(6)
Salami Leukadas	4.6	-	-	-	7.36	4.91	3.84	-
Salami Thassou	4.58	-	6.04	-	6.15	3.08	3.04	-
Salami Hellenic	4.57	-	6.06	-	6.23	3.11	3.06	4.05
Country Sausage	5.70	-	7.51	5.55	6.59	6.32	5.56	5.88
Salami aeros N+	4.33	-	4.88	3.33	-	4.31	4.11	3.89
Salami aeros T+	5.65	-	-	5.11	-	3.40	3.15	3.92
Salami aeros Y+	5.01	-	6.26	5.26	7.85	5.43	4.50	4.88
Salami aeros A+	4.81	0.83	-	5.19	3.39	5.18	3.60	-
Salami aeros BI+	4.71	-	4.06	1.12	-	-	-	-
Salami Thassou A+	4.66	0.76	5.19	3.39	5.18	3.60	-	-
Salami Pikantiko+	4.57	0.91	7.12	5.51	5.91	3.54	-	-
Salami Gravias+	5.00	0.67	5.74	4.69	5.15	-	-	-
Salami Bio+	5.11	-	7.08	-	-	-	-	-
Salami PI+	5.60	0.75	7.41	5.87	6.19	-	-	-
Sausage Leukadas*	6.15		7.51	6.50	8.99	6.03	5.20	6.06
Mean	4.92	0.78	6.05	4.32	6.18	4.13	3.89	4.52
Sd	0.45	0.09	1.09	1.49	0.91	1.09	0.93	0.86
Max	5.70	0.91	7.51	5.87	7.85	6.32	5.56	5.88
Min	4.33	0.67	4.06	1.12	5.15	3.08	3.04	3.89

[1] Each brand of sausage from both factories or shops was analysed in duplicate on two occasions over the course of a year
Samples from factories are indicated with a +, Samples from shops are not marked
Samples from island of Leukada are indicated with an *

(1) PCA (Oxoid), incubated at 25° C for 3 days
(2) Rose Bengal Chloramphenicol Agar (Oxoid), incubated at 25° C for 5 days
(3) MRS Agar (Oxoid), incubated at 25° C for 3 days
(4) Mannitol Salt Agar (Oxoid), incubated at 32° C for 3 days
(5) Baird Parker Agar (Oxoid), incubated at 37° for 1 day
(6) Schleifer and Kramer Agar (1980), incubated at 32° C for 3 days

(average count, $\log_{10}$4.52 c.f.u. g^{1}, range 3.89-5.88, st. dev. 0.86). Mannitol salt agar gave the next highest counts (average,$\log_{10}$4.13 c.f.u.$g^{-1)}$ and the medium of Baird-Parker (1962) the lowest counts. As there was such a range in counts with the media used to enumerate staphylococci, no attempt has been made to correlate these numbers with the pH of sausages.

It is unusual to attempt the isolation of yeasts from fermented sausages. As can be seen from Table 4.1, however, these organisms were relatively abundant in all samples examined.

The results presented above agree with those of Acton and Dick (1976) who analysed with respect to pH, TVC and the counts of lactic acid producing bacteria. The pH of dry sausages was <5 and the lactobacilli counts were high (average $\log_{10}$ 6.18, range 7.85-5.15). Samelis (1992, pers. comm.) has also found that lactobacilli are the dominant bacteria of fermented Greek sausages.

Three types of salami [Thassou, aeros, and aeros (picantiko)] obtained from the dispatch department of one manufacturer were stored for 14 weeks at 3°C and duplicate samples analysed at frequent intervals. The results are presented in Table 4.2. The Thassou and aeros varieties of salami shared many common features. They both had initial water activities of 0.83 or less and pH values of 4.61 or less. The numbers of pseudomonads (counted on CFC Agar with incubation at 25°C for 3 days) were invariably less than 100 per g of sausage and the yeast counts were invariably below 1.0×10^{4} g^{-1}. With both varieties, the numbers of lactobacilli tended to increase throughout storage whereas those of staphylococci diminished. These trends contrasted markedly with those obtained with salami aeros (picantiko). With the samples of the latter variety, the initial a_w was high (0.91) and remained so throughout storage. The high a_w was correlated with a

Table 4.2 Microbiological and physicochemical changes of fermented sausages during storage at 2-3° C

Salami	Parameter	Time of storage (Weeks) 1	2	3	4	6	10	14
Thassou	Yeasts*	3.39	2.81	3.80	3.23	-	2.54	3.54
	Lactobacilli*	5.18	6.95	5.41	7.16	6.90	5.48	7.17
	Pseudomonads*	<2	<2	<2	<2	<2	<2	<2
	Staphylococci*	3.60	3.70	3.51	-	2.65	<2	<2
	pH	4.66	4.55	4.48	4.55	4.54	4.56	4.88
	aw	0.76	0.77	0.78	0.78	0.79	0.80	0.79
	Moisture %	14.72	15.43	16.38	17.65	16.23	16.81	16.54
aeros	Yeasts	3.39	2.81	3.80	3.23	-	2.54	3.54
	Lactobacilli	5.18	6.95	5.41	7.16	6.90	5.48	7.19
	Pseudomonads	<2	<2	<2	<2	<2	<2	<2
	Staphylococci	3.60	3.70	3.51	-	2.65	<2	<2
	pH	4.81	4.70	4.73	4.80	4.77	4.86	5.11
	aw	0.83	0.85	0.86	0.86	0.86	0.86	0.83
	Moisture%	17.80	21.43	21.17	24.19	22.50	22.73	21.13
aeros	Yeasts	5.51	5.54	5.34	5.49	5.44	5.99	5.81
	Lactobacilli	5.91	7.69	7.38	7.27	7.99	7.61	7.47
(pikantiko)	Pseudomonads	3.56	-	2.40	-	-	-	-
	Staphylococci	3.54	4.93	4.86	5.07	4.39	5.35	6.15
	pH	4.57	4.62	4.65	4.74	4.53	5.08	5.18
	aw	0.91	0.91	0.91	0.90	0.90	0.91	0.90
	Moisture%	26.88	27.13	25.61	25.69	26.22	25.31	24.14

* Log_{10} cfu g^{-1}. Each brand of sausage was analysed in dublicate on two occasions over the course of a year

The following media were used for isolation of organisms:

Yeasts : Rose Bengal Chloramphenicol Agar (Oxoid), incubated at 25° C for 5 days
Lactobacilli : MRS Agar (Oxoid), incubated at 25° C for 3 days
Pseudomonads : CFC Agar (Oxoid), incubated at 25° C for 3 days
Staphylococci : Mannitol Salt Agar (Oxoid), incubated at 32° C for 3 days

high moisture content (average 25.9% , *cf.* 16.14% for Thassou and 21.56% for aeros ; n=7 in all cases). The numbers of lactobacilli in aeros (picantiko) increased throughout storage as did those in the varieties Thassou and aeros. The staphylococcal counts of the former increased throughout storage also whereas they diminished in the other two varieties of salami. Additionally the yeast counts of aeros (picantiko) were invariably higher than those in the other two varieties. The changes in pH are worthy of comment also. There was a slight alkaline drift in the pH values of Thassou (0.22 of a pH unit) and a more pronounced drift (0.61) in aeros (picantiko). It is tempting to speculate that these trends may be due to the levels of yeast contamination - the higher the contamination the greater the alkaline 'drift - and their possible involvement in the degradation of lactate and acetate of bacterial origin.

Throughout the survey summarised in Table 4.1 and the storage trials (Table 4.2), organisms growing on Mannitol Salt Agar, Baird-Parker medium and the medium of Schleifer and Kramer (1980) were selected and purified by repeated subculture on the medium used for their isolation. Additionally strains of *Staphylococcus* were isolated from raw materials used in sausage production, sausage making equipment and machinery as well as the hands of operatives. Once purified an organism was Gram stained and tested for catalase. Gram-positive, catalase-positive cocci were immediately characterised by the API Staph system. Two stock cultures of *Staphylococcus carnosus* (TM-300 and DM-20501) were included as controls. An isolate was identified only if it achieved a probability score of >90%. A total of 100 isolates achieved this score. Their identities are given in Table 4.3. This Table shows that 13 species of *Staphylococcus* were isolated in this survey, the major isolates being *St. aureus* (23), *St. carnosus* (17), *St. hominis* (16) and

Table 4.3 The identity* of *Staphylococcus* spp. isolated from Greek fermented sausages, sausage raw material and equipment, machinery or personnel of the sausage producing factories.

Staphylococcus*	Source				
	1	2	3	4	5
carnosus(A)	17				
hominis(H)	8	5	1	2	
xylosus(A)	7	3		2	
epidermitis(H)	2	2			3
warneri(H)	3	2		1	
saprophyticus(H)	1	3	1	1	1
simulans(H)	1	2			
intermedius(H)	1	1			
capitis(H)		2			
scuiri(A)		2			
cohnii(H)	1	1			
haemolyticus(H)		1			
aureus(H/A)	5	8	2	4	4

* In general, isolates of *Staphylococcus* spp. were randomly selected from MASA agar and Baird Parker (1962) media.

Organisms characterized with the API system, and the identification had propability of > 90%

Two stock cultures of *St. carnosus*, strains TM-300 and DM-20501 from Dr.F.Gotz (Lehrstuhl fur Mikrobiologie der Techn. Universitat Munchen, 8 Munchen, Arcisstrabe 21, BDR) were used as controls

(A) principaly of animal origin: (H) principaly of human origin (Richardson, et al. 1992)

1 Fermented sausages
2 Sausage raw material
3 Equipment (sampling by using cotton swabs)
4 Machinery (sampling by using cotton swabs)
5 Personnel (sampling by using cotton swabs)

St. xylosus (12). Judging from the results presented in Table 4.3, *St. aureus* was a common contaminant of sausages, ingredients, machinery and personnel. It needs to be stressed that these results may reflect a bias at the time of colony selection from the Baird-Parker (1962) medium used to isolate this organism. Such bias did not operate in the selection of the colonies of the other species of Staphylococcus. It was notable, therefore, that *St. carnosus* was isolated only from sausages whereas the other coagulase-negative members of this genus were more generally distributed in sausages, ingredients and on machinery.

Discussion

Table 4.1 lists the physicochemical and microbiological characteristics of the fermented sausages included in this study. All sausages were of the salami type or, in other words, the dry variety of sausage. Some samples were taken directly from the factory and others from shops in the metropolitan area of Athens. In practice the survey included sausages produced by the 7 companies which share *ca.* 90% of the market for this commodity in the shops in Athens. It is evident from this Table that in all cases the counts for lactobacilli were larger than those for staphylococci.

Moreover, the results in Table 4.2 showed a faulty "increase" in counts of lactobacilli during storage. Indeed the fluctuation of the size of Lactic acid bacteria during storage should not be interpreted as an increase of their contribution to the microbial flora. This was probably due to the great variation among the samples of the same lot. This contrasts markedly with the staphylococcal counts obtained with salami Thassou and salami aeros. In both of these the staphylococcal counts diminished during storage. This was not the situation, however, with salami aeros (picantiko). In this instance the counts of staphylococci tended to increase with time. Other features indicated that the attributes of this particular sample of sausage did not conform to the generally accepted descriptions of a dry sausage. The pH was within accepted levels but the a_w was too high. It is evident from Table 4.2 that the pseudomonad counts indicated also that the sample of picantiko was unusual. The pseudomonad counts in the first week was $> 1.5 \times 10^3$ g^{-1} in picantiko but $< 1.0 \times 10^2$ g^{-1} in the other two samples.

In addition to the sausage samples, staphylococci were

isolated from raw material taken from factories, swabs of equipment and personnel. MASA agar and Baird-Parker medium were used for this purpose.

A total of 100 isolates of staphylococci were characterised by the API Staph method. The identification of the isolates is given in Table 4.3. It needs to be stressed that the selection of isolates of *Staphylococcus aureus* was subjective in the sense that black-colonies surrounded by a precipitate were deliberately selected from Baird-Parker medium, purified and identified. The results suggest that this species is widely distributed in fermented sausages and in sausage producing factories. Whether or not their numbers pose a health problem cannot be ascertained from the results of this study because of the biased selection of isolates.

No selection of colony form occurred during the selection of the other isolates. A total of 13 species of *Staphylococcus* were identified in this study. It is noteworthy that *St. carnosus* was the most common isolate after *St. aureus*. *Staphylococcus xylosus* and *St. hominis* were both isolated on a number of occasions. If the origin of species in the recent publication by Richardson *et al.* (1992) is accepted, then it would appear that the majority of isolates were apparently of human origin (Table 4.3).

As was noted in the Literature Review on the microbiology of fermented sausage, little attention has been given until recently to the occurrence of staphylococci and their activity in salami-dry sausage. The present study has shown for the first time that *St. carnosus* is common in Greek fermented sausages. In practice this species was not isolated from every sample of sausage included in this study. Even so when the present results are compared with those of Seager *et.al.* (1986), it might be concluded that this species is much more common in Greek sausages than in those imported into

the U.K. from Europe. As Greek law prohibited the use of starter cultures until 1988 - after the present study was completed - one might take the present observations as evidence that the "back slop" technique used traditionally in Greece to inoculate sausages favoured the selection of this species. Further studies need to be done to establish the validity of this contention.

Table 4.4 summarises the results of this survey as well as those of the only other comprehensive survey done so far (Seagers *et al.* 1986). This summary shows that a fairly common range of *Staphylococcus* spp. are found in fermented sausage in Europe. Some of the species (*St. carnosus* , *St. hominis* , St. xylosus, *St. epidermidis*, *St. saprophyticus*) have been found more frequently than others. Table 5 of the Literature Review (p 26) summarises many studies of the contamination of minced beef, pork and chicken as well as products these with staphylococci. There is a very close similarity between the list of species in the Table 5 and those in Table 4.4. Indeed the main difference is the low incidence of isolation of *St. carnosus* listed in Table 5 of the Literature Review. This was also the case in a very recent survey in Greece. Kotzakidou (1992) found that in Pasturma, an intermidiate moisture meat product not fermented, *St. carnosus* was only the 4% of these isolates classified as staphylococci. This would lead one to suggest that the actual fermentation of sausages provides uniquely selective conditions for *St. carnosus* but ones that do not favour the growth of the other species of this genus.

Table 4.4 Identities of *Staphylococcus* spp. isolated from fermented sausages by different workers

Staphylococcus	Source	
	This work	Seager *et al.* (1986)
carnosus	17*	+[a]
hominis	16	+
xylosus	12	+
epidermidis	7	-
saprophyticus	7	+
warneri	6	+
simulans	3	+
intermedius	2	-
capitis	2	+
scuiri	2	+
haemolyticus	1	-
hypicus	-	+
gallinarum	-	+
caprae	-	+
aureus	23	+

* Number of isolates

[a] Other authors did not give the number of isolates of each species

Chapter 5

Meat fermentation in the laboratory

Introduction

The literature on fermented sausages was reviewed on pp 21-35. This part of the thesis was concerned with the fermentation of sausage meat made and stored in the laboratory. This work was pioneering in the sense that the Greek literature does not include any reference to similar studies.

It is obvious that fermented sausages can be considered to be solid substrates. For this reason the fermentation may be considered to be a solid-state one. Indeed it has been emphasized in recent studies that the flora is trapped in the sausage mix and consequently develops in "nests", usually enclosed in small cavities within the sausage matrix (Katsaras and Leistner 1987, 1988, 1991). Therefore for an understanding of this fermentation, it is essential to consider the formation of the sausage network, as well as the selection of the ripening flora and the biochemical changes that give special characteristics to the final product.

A comprehensive review of the literature (pp 26-28) on the development of starter cultures for cured meat products together with market analyses revealed the predominant use of lactobacilli and/or pediococci in the United States. In Europe the main bacteria used for fermentation are staphylococci, especially *Staphylococcus carnosus*, lactobacilli or pediococci (Coretti 1977; Liepe 1978; Bacus and Brown, 1981; Tetlow and Hoover 1988). As was noted in Chapter 4, the present study identified the common occurence of *St. carnosus* in Greek sausages. In this study *Lactobacillus plantarum* and *Staphylococcus carnosus* were used. These were inoculated in meat in order to examine the process of fermentation. In Chapter 6 the behaviour of these two microorganisms either alone

or in combination was studied during growth in broth cultures. A notable finding of this phase of my study was the apparent conversion of lactate to acetate as a result of environment-induced changes in the fermentation of these organisms. In this phase of the study the use of starter cultures in a sausage mix prepared in the laboratory was attempted. The primary objective was to examine the effect of the starter cultures used above on the progress (physicochemical characteristics) of the fermentation of sausages in comparison with the fermentation due to the natural flora.

Fermentation has been represented as a set of overall reactions involving carbohydrate, protein and lipid degradation, and a set of simple equations characterising the kinetics of formation of end products have been established (Demeyer and Verplaetse 1985). It is well known that the level and the type of carbohydrates, the nitrite level, the redox potential, pH, a_w, are some of the factors of equal importance to the choice of the microorganisms that are used as starter cultures (Acton *et al.* 1977; Klettner and List 1980). For instance glucose and sucrose added to sausages caused similar rates of acid accumulation during fermentation (Olsen 1985). However, a lower pH was attained when lactose has used instead of glucose or sucrose (Demeyer and Verlaetse 1985) in fermentations with starter cultures. Skjelkvale and Tsaberg (1974) indicated that the ripening of salami sausage takes a normal course without the addition of nitrite, glucono-delta-lactone (GDL) or starter culture and the microflora was very similar in studies with and without nitrite.In this study sucrose and glucose were added at concentrations of 0.48% and 0.96% respectively. A nitrite concentration of 0.01% (w/w) was used.

Among the most important extrinsic factors for correct fermentation are relative humidity and the air velocity in the ripening rooms, as well as the time these

parameters are applied to the product during the ripening process (Bacus 1986). In this study the air velocity was examined. Air circulation, however, is used by some manufactures but not by all.

Three different types of packaging systems were used for the fermentation in this study. Beakers were used in order to compare my results with those of others who have used this method. The use of natural casing in the fermentation is the most common way of making fermented sausages in Greece and elsewhere. These were used in this study also. The plastic bags were used in order to examine the effect of a substitute for casing in the fermentation procedure.

The objectives of this study were as follows.

1. To simulate manufacturing methods in order to study the fermentation of sausage meat under laboratory conditions.
2. To study the growth of *Lactobacillus plantarum, Staphylococcus carnosus* and a natural flora in sausage mixes under controlled conditions.
3. To identify the differences, if any, between fermented sausages made by using natural flora and starter cultures.

Materials and Methods

The materials and methods are given in detail in the section C (pp.44-45), Materials and Methods.

Results

A synopsis of the experiments done in this part of the study is given in Table 5.1. The initial microbial counts of the ingredients used for the preparation of

Table 5.1 The experimental parameters (organism, packaging method, air circulation) used in this study

(1)	(2)	(3)	(4)	(5)	(6)
1	a	beaker	no	5a.1	5a.7
	b	beaker	no	5b.1	5b.9
2	a	beaker	no	5a.2	5a.7
	b	beaker	no	5b.2	5b.9
3	b	beaker	no	5b.3	5b.9
4	b	beaker	no	5b.4	5b.9
5	a	casing	no	5a.3	5a.8
	b	casing	no	5b.5	5b.10
	c	casing	no	5c.1	5c.4
	d	casing	no	5d.1	5d.4
6	a	casing	no	5a.4	5a.9
	b	casing	no	5b.6	5b.11
7	a	casing	yes	5a.5	5a.10
	b	casing	yes	5b.7	5b.12
	c	casing	yes	5c.2	5c.5
	d	casing	yes	5d.2	5d.5
8	a	plastic		5a.6	5a.11
	b	bags	no	5b.8	5b.13
	c	under		5c.3	5c.6
	d	vaccum		5d.3	5d.6

(1) Experiment number
(2) Fermentation by natural flora or starter cultures
(3) Method of packaging
(4) Air movement
(5) Microbiological analysis shown in Tables 5a.1-5d.3
(6) Physicochemical analysis shown in Tables 5a.7-5d.6

a: Fermentation by natural flora
b: Fermentation by *Staphylococcus carnosus* and *Lactobacillus plantarum*
c: Fermentation by *Staphylococcus carnosus*
d: Fermentation by *Lactobacillus plantarum*

sausages are given in Table 5.2. It was notable that, with the exception of Enterobacters, the level of microbial contamination of pork with an initial pH of 6.4 was larger than that of beef or pork of pH 5.5 and 5.8 respectively. These results are in accord with those discussed in Chapter 3.

Microbiological analysis

The changes of total viable counts (isolated on Plate Count Agar), pseudomonads (isolated on CFC medium) lactic acid bacteria (isolated on MRS medium), staphylococci (isolated on Mannitol Salt Agar), yeasts (isolated on Rose Bengal Chloramphenicol Agar) and Enterobacteriaceae (isolated on VRBG Agar) during fermentation of sausage by the natural flora (Tables 5a.1, 5a.2, 5a.3, 5a.4, 5a.5, 5a.6), starter cultures (Tables 5b.1, 5b.2, 5b.3, 5b.4, 5b.5, 5b.6, 5b.7, 5b.8b) *Staphylococcus carnosus* (Tables 5c.1, 5c.2 and 5c.3) and *Lactobacillus plantarum* (Tables 5d.1, 5d.2 and 5d.3) are discussed in detail below.

As judged by the Total Viable Counts (plate Count Agar, 25° C for 3 days) the sausage mixes at the time of preparation contained 10^5 - 10^6 organisms g^{-1} (Table 5a.1-5d.3). The various selected media used in this study demonstrated that lactic acid bacteria were commonly the dominant organisms. The following, in descending order of prevalence, were present also: staphylococci, pseudomonads, yeasts and enterobacteria (Tables 5a.1 - 5d.3).

In the majority of cases there was a gradual decline in the number of pseudomonads during the incubation of the sausage mixes. Judging from the results obtained from the survey of salami discussed in Chapter 4 (see Table 4.2) one might have anticipated that these organisms would have shown a marked decline in numbers. In practice a drop of > 1.0×10^2 pseudomonads g^{-1} was found in

Table 5.2 The microbial counts and pH of the main ingredients used in sausage formulation

Count	Ingredient		
	Beef (low pH)	Pork (low pH)	Pork (high pH)
Total Viable	6.6[+]	4.5	6.1
Lactobacilli	3.5	3.4	4.5
Pseudomonads	5.4	3.7	6.5
Yeast	3.4	nd	4.8
Enterobacters	2.9	2.2	2.8
Brochothrix thermosphacta	4.5	3.3	5.1
pH	5.5	5.8	6.4

[+] Log_{10} cfu g^{-1}
nd No determined

Total Viable Counts on PCA (Oxoid)
Lactobacilli on MRS Agar (Oxoid)
Pseudomonads on CFC Agar (Oxoid)
Yeasts on Rose Bengal Chloramphenicol Agar (Oxoid)
Staphylococci on Schleifer and Kramer medium (1980)
Enterobacters on VRBG Agar (Oxoid)
Brochothrix thermosphacta on Gardner's medium

Table 5a.1 Changes in the microbial flora during the fermentation of sausages by natural flora in beaker at 23° C: 1st experiment

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)	(6)
0	4.9	3.2	4.4	5.3	2.8	1.2
1	5.2	2.5	4.9	4.2	2.8	1.3
2	6.9	2.5	6.7	4.9	4.3	1.7
4	8.04	2.2	7.9	4.6	5.6	3.1
7	6.7	3.9	6.2	5.7	5.7	4.1
9	6.9	2.9	6.8	5.2	4.3	4.01

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days
(6) Enterobacteria counts on VRBG Agar (Oxoid) incubated at 37° C for 24 hours

Table 5a.2 Changes in the microbial flora during the fermentation of sausages in beaker by natural flora at 23o C: 2nd experiment

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)	(6)
0	5.1	4.4	4.5	4.4	3.8	1.4
1	6.2	3.8	6.8	5.5	5.01	2.1
2	7.7	3.6	6.9	6.9	4.7	4.6
3	7.9	4.7	7.1	6.8	5.3	5.3
4	8.1	4.9	7.6	6.5	5.1	5.4
7	7.4	4.2	6.9	6.6	4.6	5.1
9	8.2	-	-	-	-	5.1

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days
(6) Enterobacteria counts on VRBG Agar (Oxoid) incubated at 37° C for 24 hours

Table 5a.3 Changes in the microbial flora during the fermentation of sausages in casings by natural flora incubated without air circulation at 23° C: 1st experiment

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)	(6)
0	6.9	5.7	6.2	3.5	4.4	1.5
1	9.1	5.6	7.7	5.4	5.01	2.4
2	8.4	5.8	7.9	7.01	4.4	3.01
3	8.1	5.1	7.9	6.2	5.01	3.1
7	8.5	5.3	8.2	8.2	6.2	2.1
9	7.9	4.7	8.01	7.01	5.5	3.4

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days
(6) Enterobacteria counts on VRBG Agar (Oxoid) incubated at 37° C for 24 hours

Table 5a.4 Changes in the microbial flora during the fermentation of sausages in casings by natural flora incubated without air circulation at 23° C

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)
0	6.3	-	6.1	4.3	4.6
1	7.4	6.2	7.1	5.01	4.7
2	9.01	-	8.9	7.3	6.4
3	7.6	6.2	8.01	5.8	6.8
4	8.4	5.8	8.2	-	7.1
6	8.7	5.2	8.7	-	7.3
7	8.7	4.9	8.3	7.3	7.4
9	-	4.8	7.9	6.7	7.2

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days

Table 5a.5 Changes in the microbial flora during the fermentation of sausages in casings by natural flora incubated with air circulation at 23° C

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)	(6)
0	6.01	5.8	5.2	5.3	4.4	3.2
1	6.9	5.9	7.2	-	4.5	3.4
2	7.5	5.6	8.2	5.01	4.5	2.9
3	8.01	5.1	7.9	6.2	4.3	2.9
6	8.2	7.6	7.6	-	5.3	-
7	-	-	-	7.7	-	-
9	7.2	6.8	6.8	7.1	4.8	1.5

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days
(6) Enterobacteria counts on VRBG Agar (Oxoid) incubated at 37° C for 24 hours

Table 5a.6 Changes in the microbial flora during the fermentation of sausages in plastic bags under vacuum by natural flora at 23° C

Time (days)	Log_{10} cfu g_{-1} (1)	(2)	(3)	(4)	(5)	(6)
0	5.5	5.6	4.6	4.7	4.1	1.5
1	8.01	5.8	7.2	6.7	6.2	3.5
2	8.6	6.4	8.3	7.6	6.01	4.6
4	8.2	5.6	8.01	7.8	5.8	2.7
7	8.7	-	9.4	7.1	6.2	4.6
9	8.3	6.5	9.2	7.4	6.2	4.6

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days
(6) Enterobacteria counts on VRBG Agar (Oxoid) incubated at 37° C for 24 hours

Table 5b.1 Changes in the microbial flora during the fermentation of sausages in beaker by starter cultures of *Staphylococcus carnosus* and *Lactobacillus plantarum* at 23° C, 1st experiment

Time (days)	Log10 cfu g-1 (1)	(2)	(3)	(4)	(5)	(6)
0	4.9	3.2	4.4	5.3	2.8	1.2
1	6.9	2.7	6.7	7.01	2.9	2.8
2	7.2	2.9	6.8	6.9	3.9	4.9
4	7.1	3.01	6.9	6.8	4.3	6.6
7	8.01	2.5	7.9	5.4	4.7	4.1
9	7.9	1.5	7.5	5.5	2.5	3.01

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days
(6) Enterobacteria counts on VRBG Agar (Oxoid) incubated at 37° C for 24 hours

Table 5.2b Changes in the microbial flora during the fermentation of sausages in beaker by starter cultures of *Staphylococcus carnosus* and *Lactobacillus plantarum* at 23° C: 2nd experiment

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)	(6)
0	5.1	4.4	4.5	4.4	3.8	1.4
1	5.9	2.7	5.3	-	4.7	2.0
2	8.2	4.4	8.01	6.9	4.9	4.01
3	8.01	4.01	8.3	6.7	4.9	-
4	8.3	4.4	8.01	7.5	5.6	3.6
7	8.2	3.6	7.9	8.01	4.5	-
9	-	-	7.7	-	5.01	3.2

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days
(6) Enterobacteria counts on VRBG Agar (Oxoid) incubated at 37° C for 24 hours

Table 5b.3 Changes in the microbial flora during the fermentation of sausages in beaker by starter cultures of *Staphylococcus carnosus* and *Lactobacillus plantarum* at 23° C: 3rd experiment

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)	(6)
0	4.9	6.7	4.4	5.3	2.8	1.2
1	6.2	6.1	5.9	7.3	3.1	3.01
2	6.1	4.6	6.7	7.7	3.4	5.01
3	-	-	-	7.1	-	5.1
4	6.6	4.4	6.4	6.6	3.6	6.7
7	8.01	3.6	7.2	7.2	4.7	4.5
9	7.9	4.01	8.3	-	5.6	3.01

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days
(6) Enterobacteria counts on VRBG Agar (Oxoid) incubated at 37° C for 24 hours

Table 5b.4 Changes in the microbial flora during the fermentation of sausages in beaker by starter cultures of *Staphylococcus carnosus* and *Lactobacillus plantarum* at 23° C

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)	(6)
0	5.1	3.8	4.5	-	3.8	-
1	9.5	5.5	6.8	-	3.9	-
2	9.1	6.5	7.4	-	4.2	-
3	8.3	7.1	7.01	-	4.4	-
4	8.1	-	7.7	-	3.6	-
7	7.4	3.6	7.7	-	5.1	-
9	-	-	7.9	-	4.4	-

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days
(6) Enterobacteria counts on VRBG Agar (Oxoid) incubated at 37° C for 24 hours

Table 5b.5 Changes in the microbial flora during the fermentation of sausages in casings by starter cultures of *Staphylococcus carnosus* and *Lactobacillus plantarum* incubated without air circulation at 23° C: 1st experiment

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)	(6)
0	6.9	5.7	6.2	3.5	4.4	1.5
1	9.5	5.6	8.9	7.9	5.4	2.3
2	9.1	5.6	7.9	7.3	-	2.5
3	8.3	5.4	8.2	-	6.7	1.0
6	8.4	-	-	-	-	-
7	9.9	3.4	8.4	-	-	2.6
9	8.3	-	8.3	7.0	7.0	2.6

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days
(6) Enterobacteria counts on VRBG Agar (Oxoid) incubated at 37° C for 24 hours

Table 5b.6 Changes in the microbial flora during the fermentation of sausages in casings by starter cultures of *Staphylococcus carnosus* and *Lactobacillus plantarum* incubated without air circulation at 23° C: 2nd experiment

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)
0	6.3	4.6	6.1	4.3	4.6
1	9.1	5.4	7.8	8.2	3.7
2	8.6	4.8	8.2	8.2	3.4
3	8.7	4.7	8.6	8.6	3.3
7	8.7	4.6	9.4	7.9	3.4
9	8.7	4.2	9.7	8.6	5.5

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days

Table 5b.7 Changes in the microbial flora during the fermentation of sausages in casings by starter cultures of *Staphylococcus carnosus* and *Lactobacillus plantarum* incubated with air circulation at 23° C

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)	(6)
0	6.01	5.8	5.2	5.3	4.4	3.2
1	7.3	5.8	7.7	7.1	4.5	3.4
2	7.5	4.1	7.6	6.9	3.8	2.9
3	7.7	5.01	7.9	6.9	4.1	2.9
6	7.5	-	-	-	-	-
9	7.2	3.01	7.8	7.1	4.3	1.6

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days

Table 5b.8 Changes in the microbial flora during the fermentation of sausages in plastic bags by starter cultures of *Staphylococcus carnosus* and *Lactobacillus plantarum* at 23° C

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)	(6)
0	5.5	5.6	4.6	4.7	4.1	1.5
1	8.4	5.6	7.9	5.8	4.8	2.5
2	9.7	6.7	9.4	6.01	5.6	4.01
4	8.4	5.9	8.3	8.1	5.3	4.8
7	8.7	-	8.7	-	6.9	4.6
9	8.6	6.9	8.6	7.2	6.7	4.3

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days
(6) Enterobacteria counts on VRBG Agar (Oxoid) incubated at 37° C for 24 hours

Table 5c.1 Changes in the microbial flora during the fermentation of sausages in casings by *Staphylococcus carnosus* incubated without air circulation at 23° C

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)
0	6.9	5.7	6.2	3.5	4.4
1	7.7	5.2	7.5	-	4.4
2	8.8	5.3	7.9	7.3	4.5
3	8.3	5.7	8.3	8.6	4.6
7	8.6	-	8.2	8.4	4.3
9	8.4	4.6	8.4	-	4.01

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days

Table 5c.2 Changes in the microbial flora during the fermentation of sausages in casings by *Staphylococcus carnosus* incubated with air circulation at 23° C

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)
0	6.01	5.8	5.2	5.3	4.4
1	6.6	5.4	7.1	6.7	4.2
2	7.5	5.5	7.8	6.8	3.8
3	7.5	5.1	8.01	6.7	3.6
7	-	-	-	7.1	-
9	7.2	2.3	7.01	-	3.8

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days

Table 5c.3 Changes in the microbial flora during the fermentation of sausages in plastic bags under vacuum by *Staphylococcus carnosus* incubated at 23° C

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)
0	5.5	5.6	4.6	4.7	4.1
1	8.3	5.9	7.2	5.8	4.8
2	8.5	6.3	8.3	6.6	5.7
3	-	-	-	7.5	-
7	8.6	-	9.3	-	5.9
9	8.7	5.7	8.6	8.01	6.01

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days

Table 5d.1 Changes in the microbial flora during the fermentation of sausages in casings by *Lactobacillus plantarum* incubated without air circulation at 23° C

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)
0	6.9	5.7	6.2	3.5	4.4
1	9.2	6.2	9.6	4.4	4.6
2	8.7	5.5	9.4	-	5.01
3	8.6	5.2	8.6	6.2	4.01
7	8.6	-	8.2	4.2	3.9
9	8.4	4.4	8.2	7.01	4.01

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days

Table 5d.2 Changes in the microbial flora during the fermentation of sausages in casings by *Lactobacillus plantarum* incubated with air circulation at 23° C

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)
0	6.01	5.8	5.2	5.3	4.4
1	7.5	5.7	7.6	6.6	4.8
2	7.6	5.5	8.01	5.5	4.5
3	7.6	5.2	8.1	6.2	4.6
7	-	-	-	7.01	-
9	7.5	2.9	7.2	7.1	5.4

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days

Table 5d.3 Changes in the microbial flora during the fermentation of sausages in plastic bags under vacuum by *Lactobacillus plantarum* at 23° C

Time (days)	Log_{10} cfu g^{-1} (1)	(2)	(3)	(4)	(5)
0	5.5	5.6	4.6	4.7	4.1
1	8.1	6.1	8.01	5.9	4.9
2	8.8	6.9	8.6	7.6	5.9
7	8.8	-	8.8	-	6.9
9	9.3	7.1	8.6	5.6	8.1

(1) Total Viable Counts on PCA (Oxoid) incubated at 25° C for 3 days
(2) Pseudomonads counts on CFC Agar (Oxoid) incubated at 25° C for 3 days
(3) Lactobacilli counts on MRS Agar (Oxoid) incubated at 25° C for 4 days
(4) Staphylococci counts on Mannitol Salt Agar (Oxoid) incubated at 32° C for 3 days
(5) Yeast counts on Rose Bengal Chloramphenicol Agar (Oxoid) incubated at 25° for 5 days

only two instances, sausages inoculated with *Lact. planatrum* or *St. carnosus* and packaged in natural casings. With 8 out of 12 sausage mixes the numbers of yeasts increased during incubation. If the numbers of pseudomonads and yeasts are used to categorise the sausage mixes examined in this phase of the study, then it is evident from a comparison with the results obtained in the survey of salami (chapter 4) that my preparation resembled salami aeros and picantiko (Table 4.2).

The major exceptions to the trend, discussed above, were noted with sausage meat vacuum packed in plastic bags. In all four experiments with this form of packaging, the pseudomonad counts increased by about one log cycle (Tables 5a.6, 5b.8, 5c.3, 5d.3).

An increase of this order was noted also with uninoculated sausage meat in natural casings incubated in moving air (Table 5a.5). The Enterobacteria counts suggested also that the plastic bag method of packaging produced a markedly different environment from that of a beaker or natural casings. Thus on the two occasions that these organisms were sought in sausage meat in plastic bags, their counts increasing by *ca.* 3 log cycles during incubation (Tables 5a.6, 5b.8). Increased numbers of enterobacteria was a feature of sausage meat in beakers but generally the increase was less than 3 log cycles (Tables 5a.1, 5a.2, 5b.1, 5b.2, 5b.3, 5b.4). With sausage meat in casings, the Enterobacteria counts diminished in three out of four experiments (Tables 5a.3, 5b.5and & 5a.5). With 17 of the 20 experiments, the numbers of yeasts in sausage meat increased during incubation. The largest increase tended to occur in sausage mix in plastic bags (Tables 5a.6, 5b.8, 5c.3, 5d.3) and the slowest in inoculated sausage meat in natural casing incubated in moving air (Tables 5a.5, 5b.7, 5c.2, 5d.2).

The lactic acid bacteria in sausage meat, whether inoculated or not with starter cultures, multiplied vigorously and attained numerical dominance in the first few days of incubation. The latter trend was a feature of samples of sausage meat which had been inoculated with *Lact. plantarum* or *St. carnosus* even though the number of organisms added had little if any effect on the initial counts of lactic acid bacteria. It was notable also that there was an inverse relationship between the initial number of lactic acid bacteria and the extent of their growth. Small initial numbers grew more extensively than large ones and vice versa. As judged from the counts obtained with mannitol salt agar (32°C for 3 days), staphylococci grew in all the sausage mixes but not to the same extent as the lactic acid bacteria (Tables 5a.1, 5b.1, 5a.3, 5b.5, 5a.5, 5b.7)

If the results obtained from this study of sausages made in the laboratory are compared to those obtained with commercial samples (Chapter 4), three generalisations can be made.

1. Vacuum packaging of sausage meat in plastic bags appeared to be an unsatisfactory method of studying sausage fermentation in the laboratory because of the growth of pseudomonads and enterobacteria (Tables 5a.6, 5b.8, 5c.3, 5d.3).

2. The beaker method - sausage meat packed in a beaker and covered with paraffin wax - appeared also to be an unsatisfactory method of simulating sausage fermentation in the laboratory even though it has been used by previous workers for this purpose (Acton *et al.* 1977). The main critisism is that this method did not inhibit the growth of enterobacteria (Tables 5a.1, 5a.2, 5b.1, 5b.2).

3. The best simulation of sausage production was obtained with sausage meat inoculated with starter organisms, stuffed into natural casings and incubated in moving air. Even with the last mentioned, the microbiology of the sausages was more akin to salami

aeros picantiko rather than to salami Thassou or salami aeros (Table 4.2) in the sense that the former harboured pseudomonads whereas the other two varieties did not. Even so the sausages made in this study shared an important attribute with all the commercial samples, namely the microflora was dominated by lactic acid bacteria with staphylococci being the next most prevalent organisms (Tables 5a.5, 5b.7, 5c.2, 5d.2).

Physicochemical results

The changes of moisture, a_w, sugar level, total soluble proteins (determined by the method of Lowry *et al.*, 1951) or exoprotein (determined by coomassie blue reagent) (Bradford 1976) and the production of lactic acid are given in Tables 5a.7, 5a.8, 5a.9, 5a.10, 5a.11, 5b.9, 5b.10, 5b.11 5b.12, 5b.13, 5c.4, 5c.5, 5c.6, 5d.4,5d.5 and 5d.6. The behaviour of bacteria in fermented sausages during the ripening process in relation to percentage of the original weight (moisture) and pH has been described by Lucke (1985a,b). He stated that the development of lactic acid bacteria dominate the microbial flora within 3days while pseudomonads and coliforms die out. At the mean time the rate of moisture and pH drop are significantly higher than these occurring in naturally fermented sausages. In this study, results similar to those of Lucke were obtained (Fig.5.1a, 5.1b, 5.2a and 5.2b) in samples fermented with starter cultures or the natural flora. Figure 5.1a and 5.2a show that the moisture content of the sausage mixture decreased steadily. The pH decreased at the beginning of the fermentation process but later increased again especially with the fermentations brought about by the natural flora. The decrease of moisture and pH were more pronounced in samples fermented by starter cultures (Fig.5.1a). The pH values during a 9-days period of fermentation are given in Tables 5a.7 and 5b.9. The pH

Table 5a.7 Changes of pH during meat fermentation in beaker by natural flora at 23° C

Time (days)	pH 1st experiment	2nd experiment
0	6.0	5.9
1	6.1	5.9
2	6.1	5.8
3	-	5.5
4	5.8	5.2
7	5.2	4.8
9	5.1	4.7

Table 5a.8 Changes in physicochemical characteristics during the fermentation of sausages in casings by natural flora incubated without air circulation at 23° C: 1st experiment

Time (days)	(1)	(2)	(3)	(4)	(5)	(6)	(7)
0	1950	6.3	649	65.0	0.97	1200	2020
1	1515	5.5	544	59.8	0.96	1580	2757
2	1137	5.2	1054	59.8	0.96	3156	3541
3	836	4.9	1273	57.8	0.95	2110	1926
4	-	-	-	46.5	0.93	-	-
6	-	-	-	39.5	0.86	-	-
7	402	5.4	771	-	0.87	767	1509
9	533	5.8	665	36.8	0.87	252	980

(1) Glucose mg $100g^{-1}$ sausage- dry matter
(2) pH
(3) Lactic acid mg $100g^{-1}$ sausage-dry matter
(4) Moisture%
(5) a_w
(6) Water soluble proteins estimated by Lowry method úg $100g^{-1}$ sausage-dry matter
(7) Water soluble proteins estimated by coomassie method úg $100g^{-1}$ sausage-dry matter

Table 5a.9 Changes in physicochemical characteristics during the fermentation of sausages in casings by natural flora incubated without air circulation at 23° C: 2nd experiment

Time (days)	(1)	(2)	(4)	(5)	(6)	(7)
0	2196	6.3	68.0	0.96	1064	1230
1	2028	6.4	67.5	0.96	1826	1425
2	-	6.2	66.6	0.96	1826	1517
3	1372	5.7	66.6	0.96	1102	2246
4	-	5.8	64.2	-	-	-
6	1153	-	-	-	1154	1324
7	1389	5.6	-	0.98	-	-
9	1040	5.7	47.6	0.94	1022	1009

(1) Glucose mg $100g^{-1}$ sausage- dry matter
(2) pH
(4) Moisture%
(5) a_w
(6) Water soluble proteins estimated by Lowry method úg $100g^{-1}$ sausage-dry matter
(7) Water soluble proteins estimated by coomassie method úg $100g^{-1}$ sausage-dry matter

Table 5a.10 Changes in physicochemical characteristics during the fermentation of sausages in casings by natural flora incubated with air circulation at 23° C

Time (days)	(1)	(2)	(3)	(4)	(5)	(6)	(7)
0	2021	6.1	735	69.0	0.96	838	1044
1	1568	6.1	1106	55.4	0.95	-	-
2	1441	6.1	1069	50.2	0.93	782	870
3	1385	5.9	998	45.5	0.89	554	607
4	-	-	-	-	-	-	-
6	1006	-	-	27.4	0.78	-	-
7	-	-	750	-	-	420	590
9	829	6.1	683	24.0	0.69	-	568

(1) Glucose mg $100g^{-1}$ sausage- dry matter
(2) pH
(3) Lactic acid mg $100g^{-1}$ sausage-dry matter
(4) Moisture%
(5) a_w
(6) Water soluble proteins estimated by Lowry method úg $100g^{-1}$ sausage-dry matter
(7) Water soluble proteins estimated by coomassie method úg $100g^{-1}$ sausage-dry matter

Table 5a.11 Changes in physicochemical characteristics during the fermentation of sausages in plastic bags under vacuum by natural flora at 23° C

Time (days)	(1)	(2)	(3)	(6)	(7)
0	2040	6.2	531	2632	1144
1	1850	6.1	830	1923	1919
2	1601	5.4	916	1912	-
4	292	5.1	955	2359	1652
7	183	5.3	808	2160	1243
9	138	5.4	-	1935	1104

(1) Glucose mg $100g^{-1}$ sausage- dry matter
(2) pH
(3) Lactic acid mg $100g^{-1}$ sausage-dry matter
(6) Water soluble proteins estimated by Lowry method úg $100g^{-1}$ sausage-dry matter
(7) Water soluble proteins estimated by coomassie method úg $100g^{-1}$ sausage-dry matter

Table 5b.9 Changes in the pH of fermented sausage meat in beakers by *Staphylococcus carnosus* and *Lactobacillus plantarum* at 23° C.

Time (days)	pH 1st exper.	2nd exper.	3rd exper.	4th exper.
0	6.0	5.8	6.0	6.1
1	6.1	5.3	5.9	6.1
2	6.0	4.8	5.9	5.5
3	-	-	-	5.5
4	6.0	4.7	5.5	5.1
7	5.6	4.6	5.4	4.2
9	5.1	4.3	4.9	4.2

Table 5b.10 Changes in physicochemical characteristics during the fermentation of sausages in casings by starter cultures of *Staphylococcus carnosus* and *Lactobacillus plantarum* incubated without air circulation at 23° C

Time (days)	(1)	(2)	(3)	(4)	(5)	(7)
0	1779	6.3	480	64.0	0.97	2920
1	1411	6.3	520	62.0	0.96	2757
2	778	6.1	578	57.8	0.96	3436
3	251	5.8	780	56.3	0.95	2881
7	167	5.0	990	42.3	0.87	1928
9	152	4.8	1040	39.8	0.87	741

(1) Glucose mg $100g^{-1}$ sausage- dry matter
(2) pH
(3) Lactic acid mg $100g^{-1}$ sausage-dry matter
(4) Moisture%
(5) a_w
(7) Water soluble proteins estimated by coomassie method úg $100g^{-1}$ sausage-dry matter

Table 5b.11 Changes in physicochemical characteristics during the fermentation of sausages in casings by starter cultures of *Staphylococcus carnosus* and *Lactobacillus plantarum* incubated without air circulation at 23° C: 2nd experiment

Time (days)	(1)	(2)	(3)	(4)	(5)	(6)	(7)
0	1997	6.3	510	67.0	0.97	1348	1483
1	1680	5.9	550	66.0	0.97	964	1624
2	1540	5.4	589	65.0	0.97	758	1138
3	855	5.1	830	65.0	0.96	1082	1185
4	716	-	-	-	-	-	809
6	-	-	-	-	0.92	-	-
7	167	5.1	950	-	0.90	1313	986
9	252	5.1	1120	47.3	0.90	1205	899

(1) Glucose mg $100g^{-1}$ sausage- dry matter
(2) pH
(3) Lactic acid mg $100g^{-1}$ sausage-dry matter
(4) Moisture%
(5) a_w
(6) Water soluble proteins estimated by Lowry method úg $100g^{-1}$ sausage-dry matter
(7) Water soluble proteins estimated by coomassie method úg $100g^{-1}$ sausage-dry matter

Table 5b.12 Changes in physicochemical characteristics during the fermentation of sausages in casings by starter cultures of *Staphylococcus carnosus* and *Lactobacillus plantarum* incubated with air circulation at 23° C

Time (days)	(1)	(2)	(4)	(5)	(6)	(7)
0	1697	6.0	68.4	0.98	2348	2583
1	1380	5.9	58.9	0.95	1846	2162
2	1340	5.9	51.9	0.93	2158	2113
3	-	5.9	48.7	0.90	1982	1885
4	816	-	-	-	-	809
7	940	5.7	32.6	0.80	1313	1098
9	844	5.7	24.6	0.72	523	741

(1) Glucose mg $100g^{-1}$ sausage- dry matter
(2) pH
(4) Moisture%
(5) a_w
(6) Water soluble proteins estimated by Lowry method úg $100g^{-1}$ sausage-dry matter
(7) Water soluble proteins estimated by coomassie method úg $100g^{-1}$ sausage-dry matter

Table 5b.13 Changes in physicochemical characteristics during the fermentation of sausages in plastic bags under vacuum by starter cultures of *Staphylococcus carnosus* and *Lactobacillus plantarum* at 23° C

Time (days)	(1)	(2)	(3)	(6)	(7)
0	1787	6.0	520	2700	2283
1	1880	5.9	646	2306	2562
2	1254	5.1	660	2658	1113
4	416	4.7	755	2282	780
7	406	4.7	879	4411	898
9	448	4.7	1020	5237	919

(1) Glucose mg $100g^{-1}$ sausage- dry matter
(2) pH
(3) Lactic acid mg $100g^{-1}$ sausage-dry matter
(6) Water soluble proteins estimated by Lowry method úg $100g^{-1}$ sausage-dry matter
(7) Water soluble proteins estimated by coomassie method úg $100g^{-1}$ sausage-dry matter

Table 5c.4 Changes in physicochemical characteristics during the fermentation of sausages in casings by *Staphylococcus carnosus* incubated without air circulation at 23° C

Time (days)	(1)	(2)	(3)	(4)	(5)	(6)	(7)
0	1573	6.1	567	66.0	0.98	1756	1633
1	1341	5.9	652	65.0	0.98	1341	1273
2	1184	5.3	675	65.0	0.98	1184	1003
3	925	5.0	677	56	0.95	925	750
7	890	5.0	890	52.0	0.94	890	780
9	560	5.3	800	28.4	0.91	560	500

(1) Glucose mg $100g^{-1}$ sausage- dry matter
(2) pH
(3) Lactic acid mg $100g^{-1}$ sausage-dry matter
(4) Moisture%
(5) a_w
(6) Water soluble proteins estimated by Lowry method úg $100g^{-1}$ sausage-dry matter
(7) Water soluble proteins estimated by coomassie method úg $100g^{-1}$ sausage-dry matter

Table 5c.5 Changes in physicochemical characteristics during the fermentation of sausages in casings by *Staphylococcus carnosus* incubated with air circulation at 23° C

Time (days)	(1)	(2)	(3)	(4)	(5)	(6)	(7)
0	1497	6.1	632	69.5	0.98	1590	1348
1	1368	6.1	755	62.0	0.96	1780	1524
2	1150	6.1	959	51.5	0.94	1900	1838
3	1020	5.9	983	45.0	0.88	1845	1685
6	-	-	-	33.0	0.80	-	-
7	970	5.8	935	-	0.79	1234	1196
9	902	6.0	891	21.1	0.79	1304	1289

(1) Glucose mg $100g^{-1}$ sausage- dry matter
(2) pH
(3) Lactic acid mg $100g^{-1}$ sausage-dry matter
(4) Moisture%
(5) a_w
(6) Water soluble proteins estimated by Lowry method úg $100g^{-1}$ sausage-dry matter
(7) Water soluble proteins estimated by coomassie method úg $100g^{-1}$ sausage-dry matter

Table 5c.6 Changes in physicochemical characteristics during the fermentation of sausages in plastic bags under vacuum by *Staphylococcus carnosus* at 23° C, without air circulation.

Time (days)	(1)	(2)	(3)	(6)	(7)
0	1569	6.1	623	2234	1253
1	1238	6.1	656	3001	1762
2	1040	5.4	853	3005	1413
3	870				
4	816	-	907	2534	1909
7	470	5.0	899	2490	2093
9	372	5.0	1143	3452	2789

(1) Glucose mg $100g^{-1}$ sausage- dry matter
(2) pH
(3) Lactic acid mg $100g^{-1}$ sausage-dry matter
(6) Water soluble proteins estimated by Lowry method úg $100g^{-1}$ sausage-dry matter
(7) Water soluble proteins estimated by coomassie method úg $100g^{-1}$ sausage-dry matter

Table 5d.4 Changes in physicochemical characteristics during the fermentation of sausages in casings by *Lactobacillus plantarum* at 23° C, without air circulation.

Time (days)	(1)	(2)	(3)	(5)	(6)	(7)
0	1608	6.3	490	0.98	788	1803
1	1233	5.6	679	0.96	375	2207
2	780	5.2	748	0.94	-	1942
4	556	5.1	705	0.88	462	1302
7	251	5.3	959	0.78	940	1728
9	195	5.4	1010	0.71	1245	1741

(1) Glucose mg $100g^{-1}$ sausage- dry matter
(2) pH
(3) Lactic acid mg $100g^{-1}$ sausage-dry matter
(5) a_w
(6) Water soluble proteins estimated by Lowry method úg $100g^{-1}$ sausage-dry matter
(7) Water soluble proteins estimated by coomassie method úg $100g^{-1}$ sausage-dry matter

Table 5d.5 Changes in physicochemical characteristics during the fermentation of sausages in casings by *Lactobacillus plantarum* at 23° C, with air circulation.

Time (days)	(1)	(2)	(3)	(4)	(5)	(6)	(7)
0	1747	6.1	560	67	0.97	1243	2466
1	1570	6.1	650	58	0.96	1094	1635
2	956	5.9	959	51.2	0.94	958	1813
3	-	5.8	880	45	0.88	1282	1218
4	816	-	-		-		
7	750	5.5	950	25	0.75	1113	880
9	625	5.8	891	23.4	0.71	890	612

(1) Glucose mg $100g^{-1}$ sausage- dry matter
(2) pH
(3) Lactic acid mg $100g^{-1}$ sausage-dry matter
(4) Moisture%
(5) a_w
(6) Water soluble proteins estimated by Lowry method úg $100g^{-1}$ sausage-dry matter
(7) Water soluble proteins estimated by coomassie method úg $100g^{-1}$ sausage-dry matter

Table 5d.6 Changes in physicochemical characteristics during the fermentation of sausages in plastic bags under vacuum by *Lactobacillus plantarum* at 23° C, without air circulation.

Time (days)	(1)	(2)	(3)	(5)	(6)	(7)
0	1650	6.2	571	0.98	1149	2081
1	1480	5.9	741	0.95	956	1462
2	1248	5.3	1034	0.93	1028	1313
3	-	5.3	1200	0.90	-	-
4	320	-	-	-	1021	729
7	290	5.0	1412	0.80	833	852
9	210	4.9	1311	0.72	831	1031

(1) Glucose mg $100g^{-1}$ sausage- dry matter
(2) pH
(3) Lactic acid mg $100g^{-1}$ sausage-dry matter
(4) Moisture%
(5) a_w
(6) Water soluble proteins estimated by Lowry method úg $100g^{-1}$ sausage-dry matter
(7) Water soluble proteins estimated by coomassie method úg $100g^{-1}$ sausage-dry matter

Figure 5.1a Changes of pH & moisture (%) during meat fermentation by starters without air circulation at 23o C.

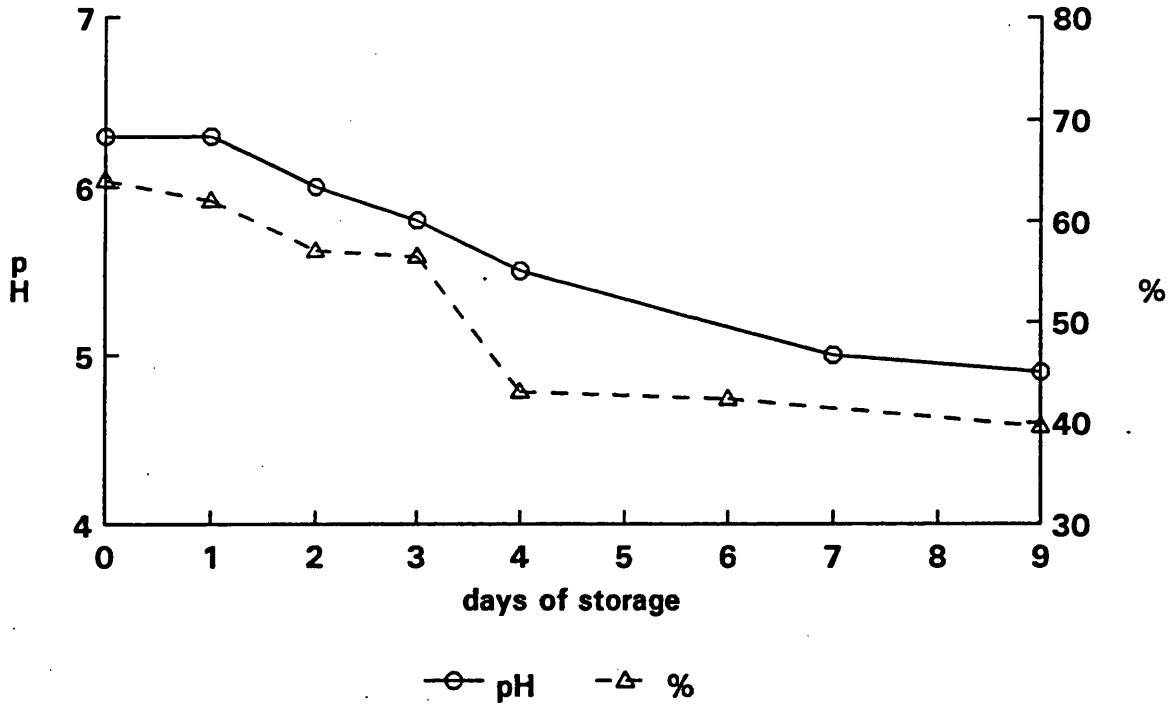
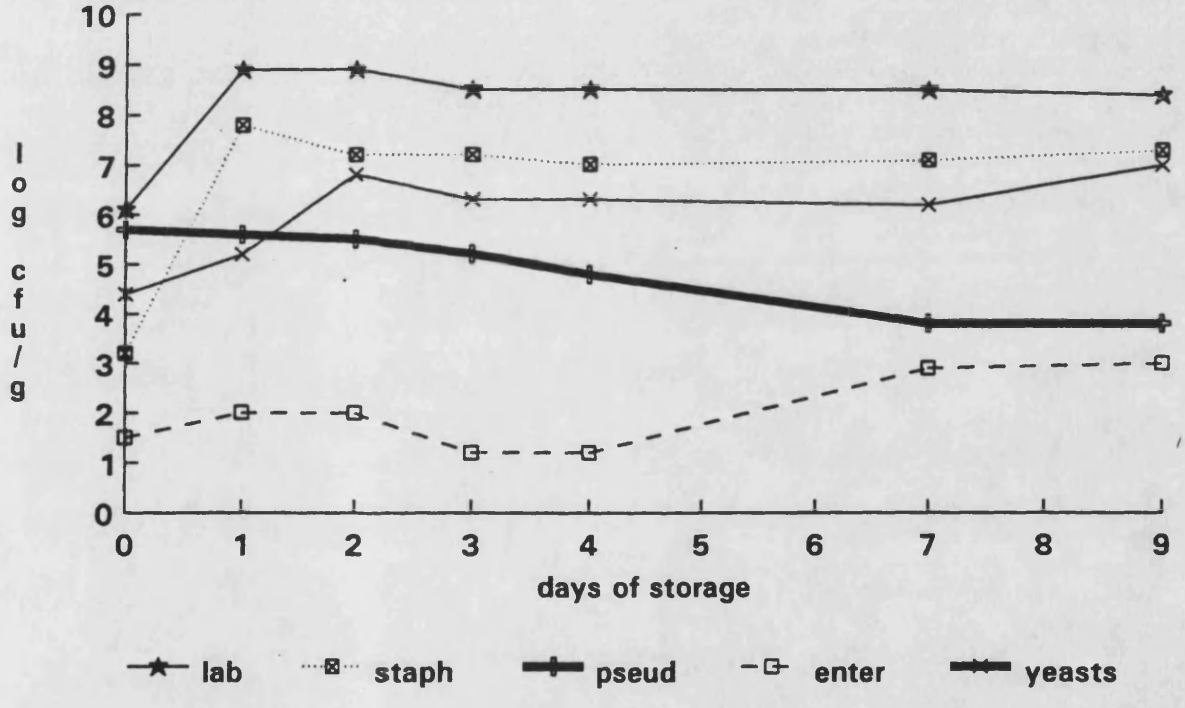


Figure 5.1b Changes of microbial flora during meat fermentation by Starters, without air circulation at 23o C



lab : Lactobacilli
staph : Staphylococci
pseu : Pseudomonads
enter : Enterobacteriaceae
yeasts : Yeasts

Figure 5.2a Changes of pH & moisture (%) during meat fermentation by natural flora without air circulation at 23oC

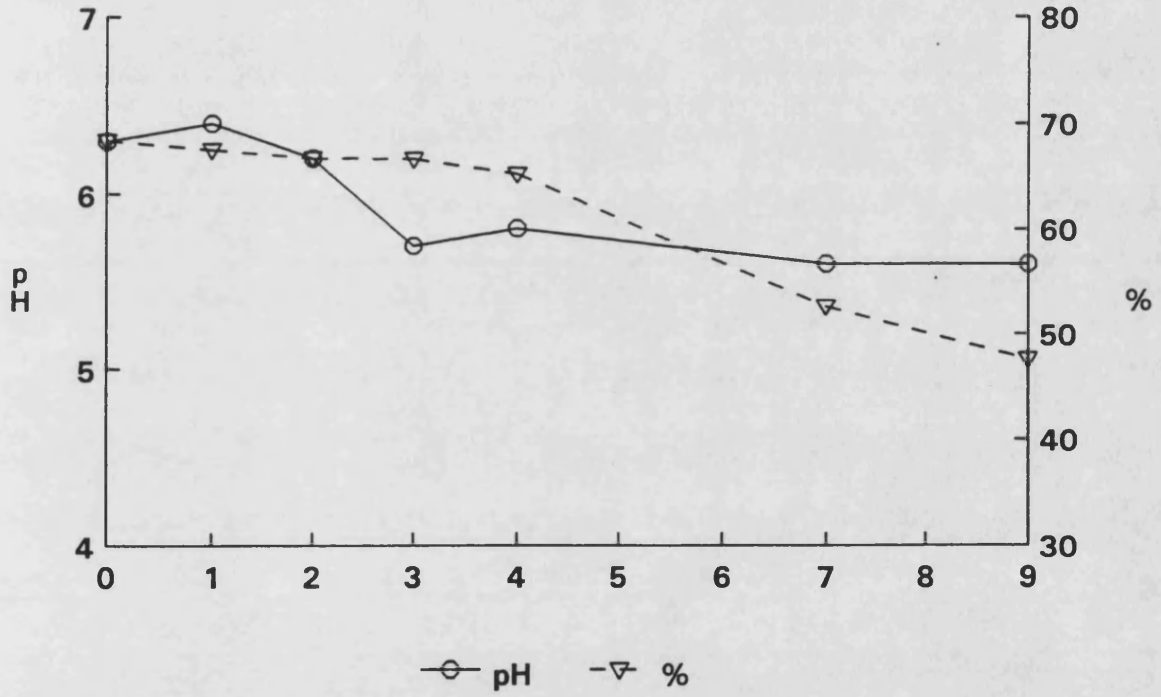
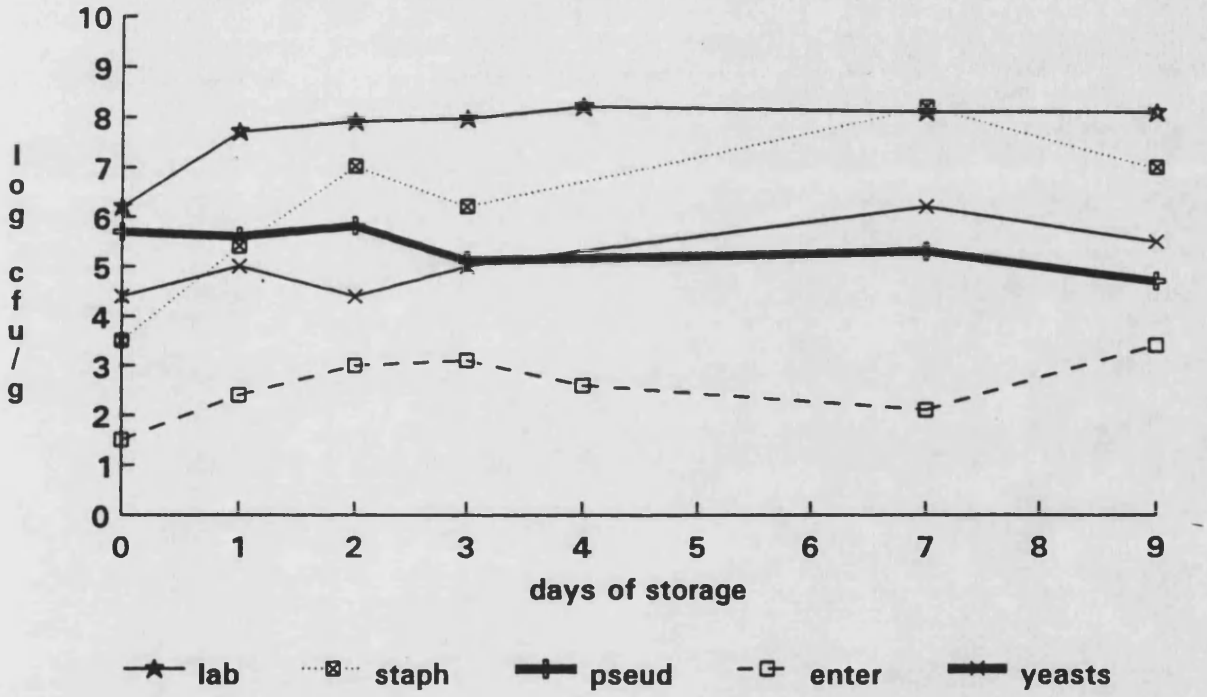


Figure 5.2b Changes of microbial flora during meat fermentation by natural flora, without air circulation at 23oC



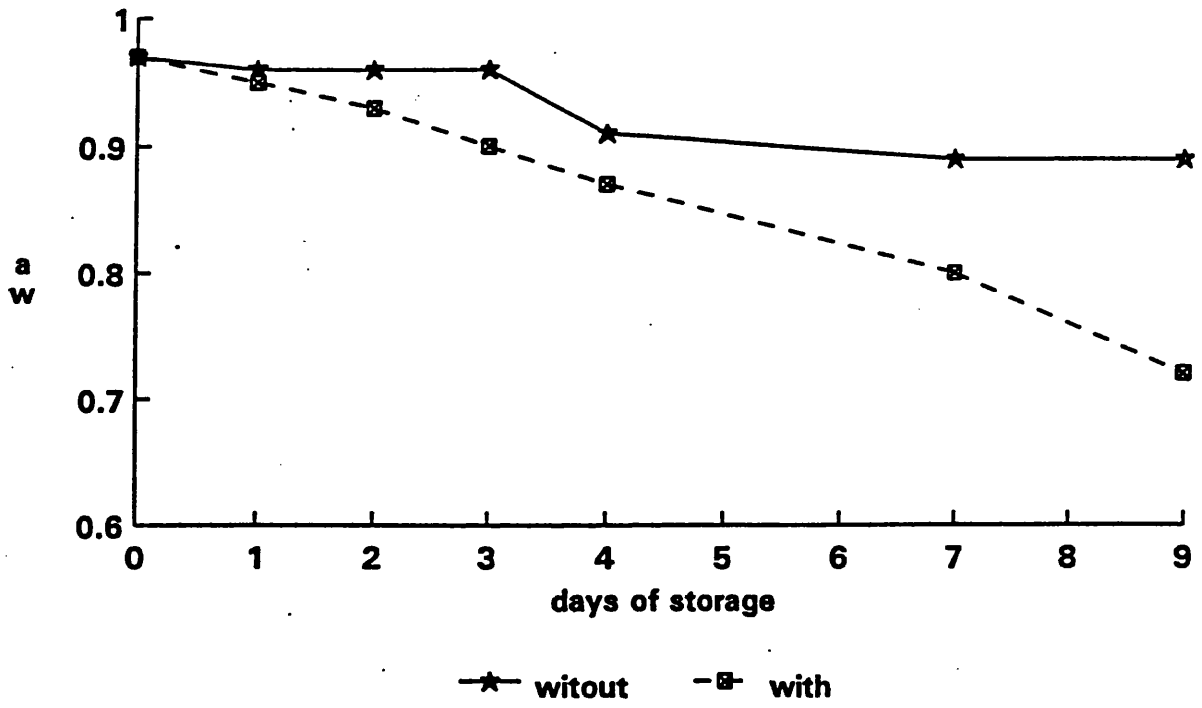
lab : Lactobacilli
staph : Staphylococci
pseud : Pseudomonads
enter : Enterobacteriaceae
yeasts : Yeasts

decreased significantly from an initial value of 6.10 to around 5.00 during fermentation for up to 7 days. Afterwards it started to drift back towards a neutral value in the case of fermentation with natural flora. The lowest pH values was obtained in those cases where *Lactobacillus plantarum* and *Staphylococcus carnosus* were used in combination and the smallest acid drift obtained when the fermentation was brought about by the natural flora.

At zero time, the water activity had an average value of 0.969 (Fig. 5.3). A gradual drop of the water activity during the first 6 days of fermentation was registered (Fig. 5.3). The final a_w and moisture content were always lower in samples in casings stored with air circulation (Fig. 5.3 and 5.4).

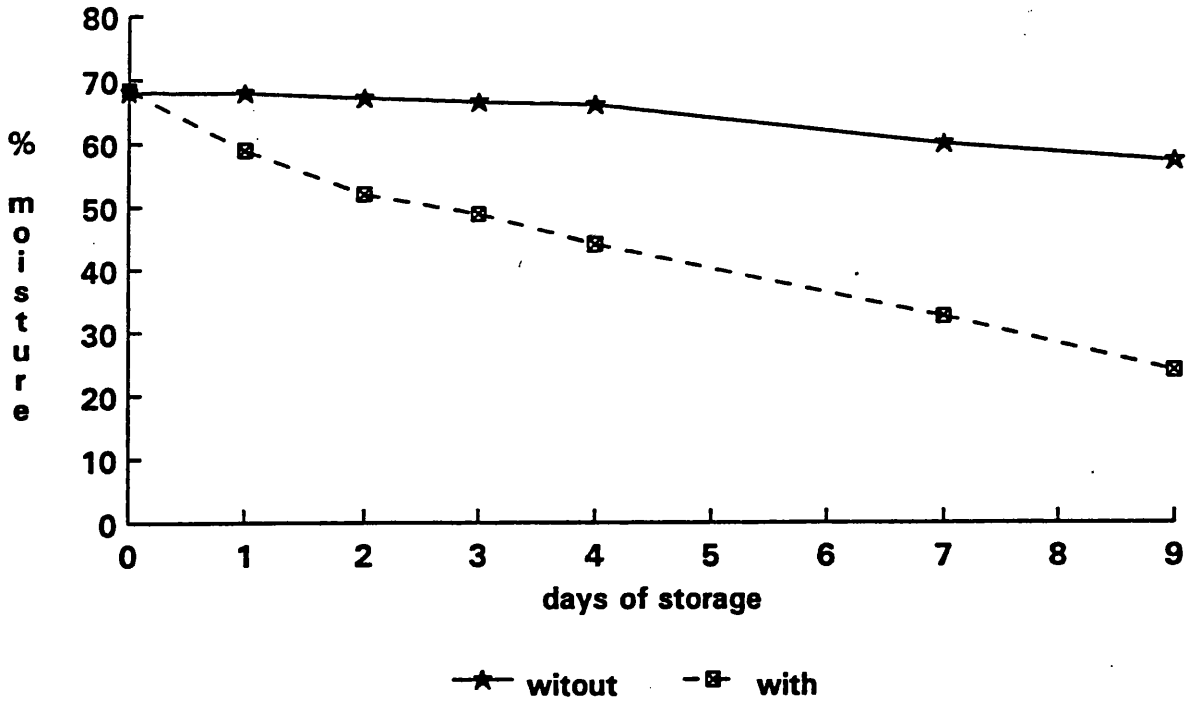
In all our samples the glucose concentration decreased with time. It was noted in some cases (Table 5.3) that the rate of glucose assimilation was higher in sausages mix fermented with starter cultures than these fermented by the indigenous lactic acid bacteria. It was also found that the lactic acid increased during the fermentation. This end-product was more pronounced in the case of sausages fermented with starter cultures. When natural flora was used for fermentation it was found that lactate increased initially followed by a decrease at the end of fermentation. In addition to lactate production the gas-liquid chromatography (Figs. 5.5 and 5.6) revealed that acetic acid and ethanol were produced also. Ethanol and carbon dioxide, in equal proportions, are produced by hetero-fermentantive microorganisms. When the 'bifidum pathway' oparates in bacteria, lactate and acetate are produced in a ratio 2:3 (Gottschalk 1979). It is well known that fermentation products other than lactic acid, such as acetic acid, ethanol, acetoin, carbon dioxide, pyruvic etc. are produced in various amounts during sausage fermentation (Strasters and

Figure 5.3 Changes of Water activity (aw) during meat fermentation with and without air circulation at 23o C



witout : without air circulation
with : with air circulation

Figure 5.4 Changes of moisture (%) during meat fermentation with and without air circulation at 23oC



witout : without air circulation
with : with air circulation

Table 5.3 Summary of the results obtained from the fermenation of meat, in casing, by natural flora and by starters (Lact. plantarum, St. carnosus and Lact. plantarum, St. carnosus in combination)

Glucose	pH	Lactate	Lactics Counts [Staph/ci counts]	Yeasts Counts	Enterobacteria (pseudomonads counts)
a. Natural Flora					
1950- 533	6.3-^5.8	649-1273* (665)#	6.2-8.0(+3)$ [3.5-8.2](+4)$$	4.4-5.01	1.5-3.4 (5.7-4.7)
2196-1040	6.3-&5.7	-	6.1-8.9(+2) [4.3-7.3](+2)	4.6-7.2	n.t (6.2-4.8)
2021-829	6.1-^5.9	735-1069 (683)#	5.2-8.2(+2) [5.3-7.1]	4.4-5.3	3.2-1.5 (5.8-6.8)
b. Inoculated with St.carnosus and Lact.plantarum					
1779-152	6.3-&4.8	480-1040##	6.2-8.3(+2) [3.5-7.0](+3)	4.4-7.0	1.5-2.6 (5.7-3.4)
1997-252	6.3-&5.1	510-1120	6.1-9.7 [4.3-8.6]	4.6-5.5	n.t (4.6-4.2)
1697-844	6.0-&5.7	-	5.2-7.9 [5.3-7.1]	4.4-4.3	3.2-1.6 (5.8-3.0)
c. Inoculated with St.carnosus					
1573-560	6.1-^5.3	567-800	6.2-8.4(+2) [3.5-8.4](+2)	4.4-4.6	n.t (5.7-4.6)
1497-902	6.1-&5.8	632-935	5.2-7.1 [5.7-7.1]	4.4-3.8	n.t (5.8-2.3)
d.Inoculated with Lact. plantarum					
1747-625	6.1-&5.5	560-891	5.2-8.1(+2) [5.3-7.1]	4.4-5.4	n.t (5.8-2.9)
1608-195	6.3-&5.4	490-1010	6.2-9.4(+2) [3.5-7.0]	4.4-4.0	n.t (5.7-4.4)

^ : Initial drop of pH followed by an increase at the end oof storage.
& : pH drop throughout the fermenation
* : Maximum lactate concentration found during the fermenation
# : Final concentration of lactate after 9 days of fermentation
##: Final lactate concentration which achieved after 9 days of fermenation
$: Periods (in days) needed for the domination of lactic acid bacteria
$$: Periods (in days) needed for the domination of Staphylococci

Fig. 5.5 Changes in volatile fatty acids in sausages during fermentation at 23° C inoculated with *Lactobacillus plantarum* and *Staphylococcus carnosus* DM-20501

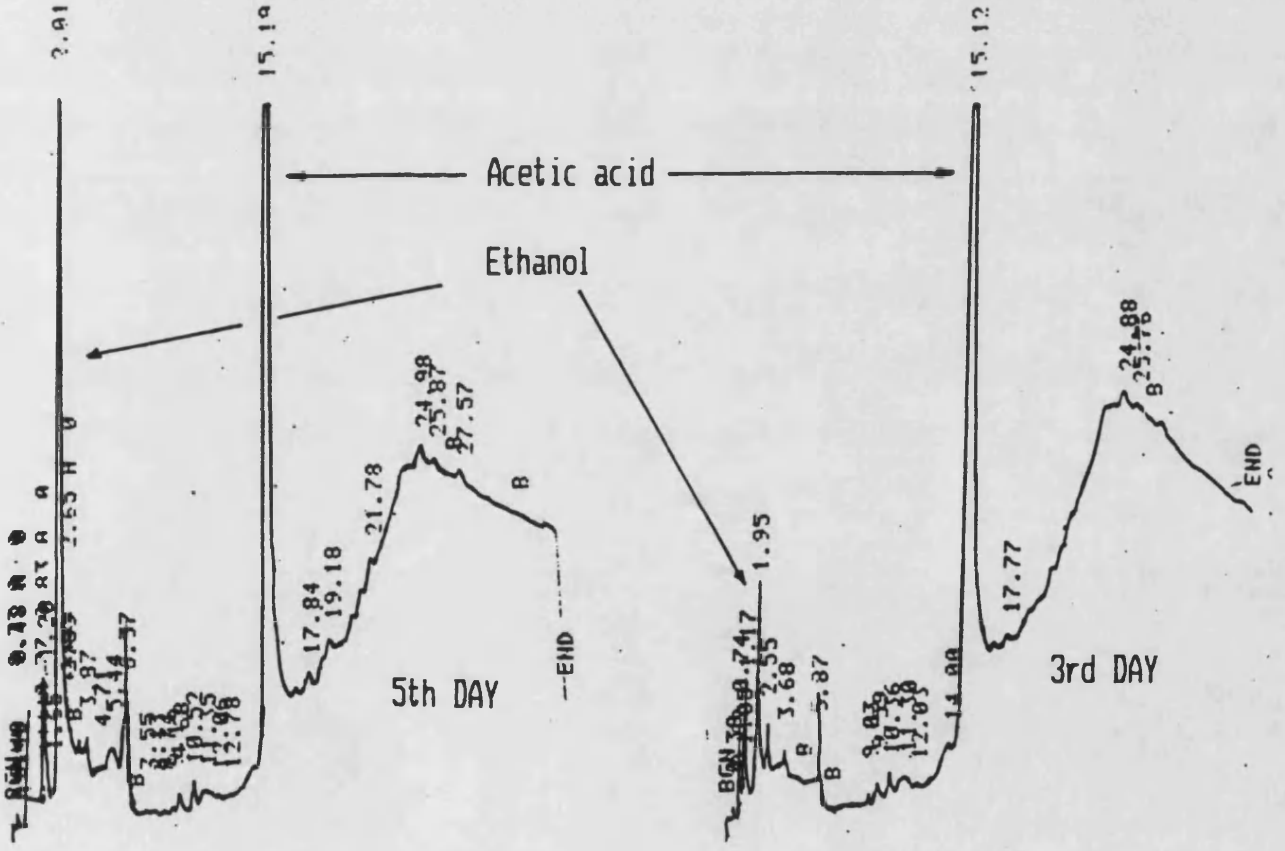
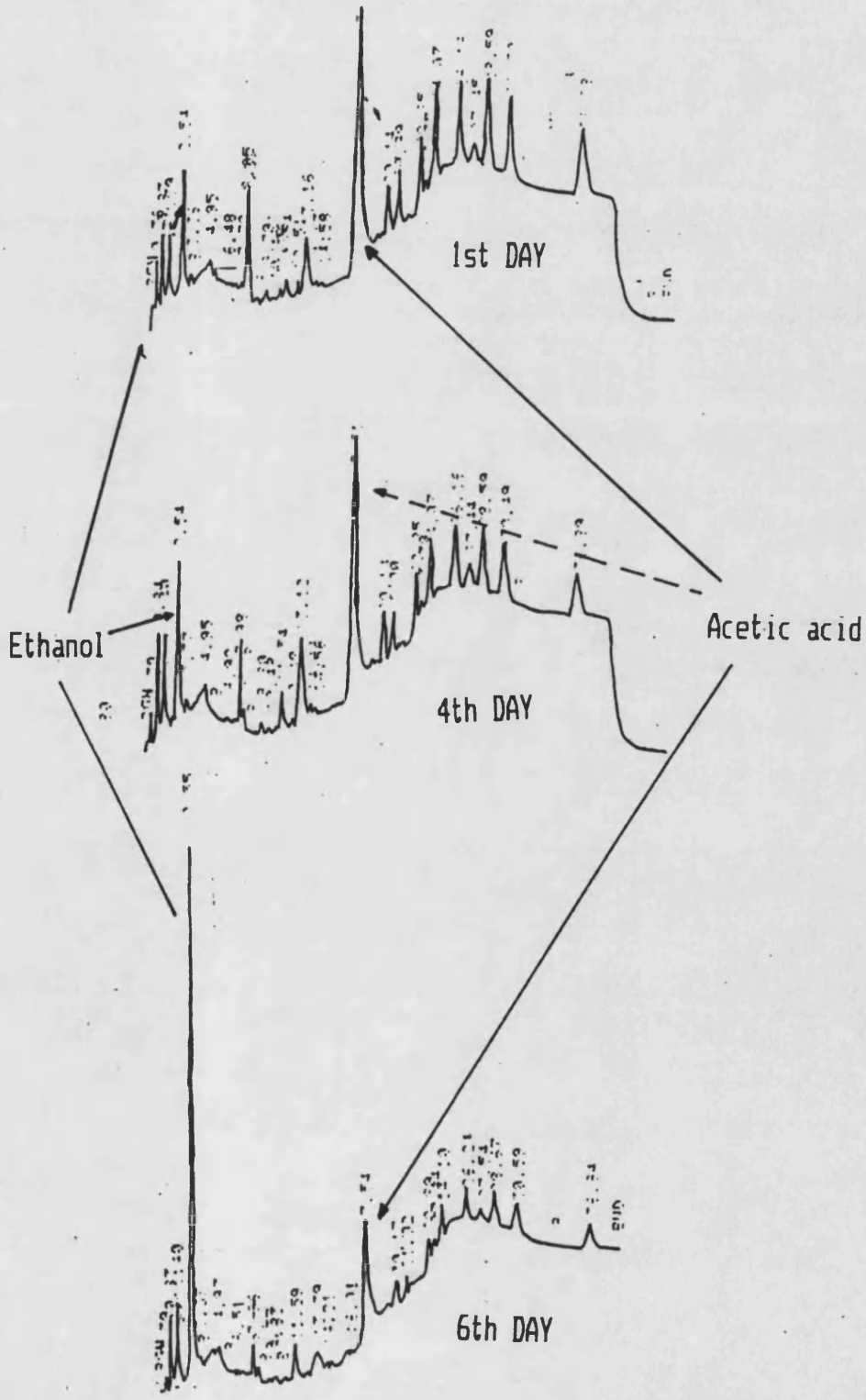


Fig. 5.6 The changes in volatile fatty acids of sausages during fermentation at 23° C inoculated with *Staphylococcus carnosus* TM-300 and *Lactobacillus plantarum*



Winkler 1963; Blumental 1972; Vandekerchove and Demeyer 1975; Demeyer 1982; Thornill and Cogan 1984; Tetlow and Hoover 1988;) and in broth cultures (Blickstad and Molin 1981). Figures 5.5 and 5.6 show the major end-products formed during the fermentation of laboratory made sausages.

In general Table 5.3 summarise the main findings of this part of the study:

1. The rate of glucose assimilation was higher in sausage mixes inoculated with starter cultures. Indeed the amount of glucose found after 9 days of fermentation in samples inoculated with starters was lower than these samples fermented with natural flora.
2. Similar results were obtained with pH.
3. The lactic acid concentration found to increase during the sauasge fermentation of samples with natural flora followed with a decrease. In samples inoculated with starter cultures there was only an increase of lactate thoughout the fermentation.
4. Lactic acid bacteria dominated during the first 3 days of the fermentation procedure in either samples (natural flora, starter cultures).
5. Yeasts, Enterobacteria and pseudomonads were controlled or inhibited in samples fermented with starter cultures. Yeasts survived or grew in samples fermented with natural flora.

DISCUSSION

It is well known that during the post-mortem period a small portion of the glycogen in meat is converted to glucose and finally to lactic acid (Lawrie 1974). As the glucose content of fresh meat (beef or pork) is of the order of 50-200 mg/100g meat (Lawrie 1974; Farber and Idziak 1982; Nychas 1984), microbial action results in only a small change in pH. For this reason fermentable carbohydrates are added to meat intended for the production of fermented sausages. In this study glucose and sucrose were added in concentrations of 0.96% and 0.48% respectively. The influence of additional sugar on pH change in meat is evident when the results in this phase of the study (Tables 5a.8, 5b.10, 5c.4, 5d.4, 5a.10, 5b.12, 5c.5, 5d.5) are compared with those in Chapter 3 (cf Table 3.7). In this latter case growth of lactic acid bacteria was not associated with an acid drift in the pH but it was when glucose and sucrose were added. On the other hand in samples fermented with starter cultures the pH dropped significant ly (Table 5.3). It is evident from Table 5.3 however that the amount of lactate produced in these samples did not correlate with the amount of glucose assimilated. This could be attributed probably to the fact that a metabolic switch from homo- to heterolactic fermentation occurs in *Lactobacillus plantarum*. Indeed the addition of glucose in static cultures (Chapter 6) as well as oxygen limitation apparently induced a heterolactic switch. This is in accord with the findings of Borch and Molin (1989). In this case the acetic acid is produced in high amounts. The accumulation of this acid could result also from further metabolism of L-Lactate by the *i*LDH enzyme. This enzyme exists in many bacteria (Garvie 1980) and converts L-lactate to pyruvate and no evidence of a reverse reaction has been found (Kandler 1983). Many reports infer that lactic acid bacteria may switch from homo- to heterofermentation (Thomas *et al.* 1979). This

matter will be discussed in detail in Chapter 6 (pp 184-187). In general the end products (lactate, acetate, volatile fatty acids, amino acids etc.) formed depend upon the composition and the size of inoculum of starter culture (Klement *et al.* 1974; Eitenmiller *et al.* 1978; Hadziosmanovic *et al.* 1979; Olsen 1985; Kato *et al.* 1985; Bacus 1986; Numata *et al.* 1988a,b), the type and the amount of carbohydrate added (Deketeleare *et al.* 1974; Acton *et al.* 1977; Olsen 1985; Lee 1987; Lois *et al.* 1987), the source and previous treatment of meat proteins (Klement *et al.* 1973; Townsend *et al.* 1980; Pezacki and Pezacka 1987), and the addition of various additives such as glucono-delta- lactone (GDL), spices etc. (Nes and Skejelvale 1982; Lee 1987).

According to Katsaras and Leistner (1991) many of these reactions (glycolysis, proteolysis, lipolysis) could occur in the fermented sausages. Indeed these workers reported that the cells of the starter cultures are immobilised in cavities or "nests" within the sausage matrix. In this case, there is an accumulation of end-products and a serious depletion of nutrients together with oxygen limitation. Murphy and Condon (1984) found that acetic acid was produced under aerobic or anaerobic condition from *Lactobacillus planatrum* in a glucose medium. In our study the lactic acid bacteria were the dominant organisms and so their contribution to the microbial products would be expected to be extremely high.

This observation raises an important question and one that does not appear to have been addressed in the literature - what substrates do lactobacilli use in normal meat?

In general it is well known that the proteolytic activity of lactic acid bacteria is weak (Kitchell and Shaw 1975; Law and Kolstad 1983). The spoilage of meat due to proteolysis or, more importantly, the development of aroma and flavour with free amino acids (FAA) and free

fatty acids (FFA) in the case of fermented sausages, cannot be attributed to their activity. In his review Bacus (1986) reported that micrococci-type bacteria have been used extensively for many years but solely in European sausages. Although micrococci/staphylococci produce extracellular proteinases (McDonald and Chamber 1966; Donham *et al.* 1988), their role in the proteolysis of fermented sausages is in dispute. The increase of free amino acids (FAA) and proteolysis in fermented sausage has been attributed to these organisms (Sajber *et al.* 1971; Bacus 1986; and a trade leaflet of Chr. Hansens Co). Although Niinivaara *et al.* (1964) did not find any differences in FAA between sausages fermented naturally or inoculated deliberately with micrococci, they suggested that the decomposition of proteins during the fermentation is the result of the activity of bacteria inoculated into sausages.

In my study the analysis of water soluble proteins with two methods (Lowry et al. 1951; Bradford 1976) was proved insufficient to draw any conlusion concerning the effect of starter cultures on the rate of proteolysis. This matter was also examined further in Chapter 6 (pp. 184-185).

Chapter 6

Studies of the factors that affect the growth and physicochemical attributes of *Lactobacillus plantarum and Staphylococcus carnosus* in broths

Introduction

The Literature Review (pp 26-28) on *Staphylococcus carnosus* and, to a lesser extent, *Lactobacillus plantarum* gave an up-to-date account of the use of starter cultures in the processing of fermented sausages worldwide.

It is well known that, in addition to preservation of a product, starter cultures are used in order to obtain a desirable aroma, flavour and texture in these types of sausage as well as control of food poisoning organisms (Borch *et al.* 1991). Judging from the literature, little attention has been paid to the contribution of *Staphylococcus carnosus* in this fermentation. Niinivaara and Pohja (1956, 1957a,b) were probably the first to show that unidentified staphylococci grew extensively during the fermentation phase of Finnish sausage. Indeed they stated that these bacteria probably contributed to the organoleptic properties of the product. This view is supported by our knowledge of the metabolic activity of these bacteria (Andres 1977; Bacus 1986).

The metabolism of lactic acid bacteria and staphylococci is affected by environmental factors which could well influence their beneficial/detrimental contribution to the organoleptic properties of sausages. Oxygen, pH, water activity, as well as the concentration of lactic acid, glucose and amino acid are reported to affect metabolic product patterns (Thomas *et al.*1979; Nychas *et al.* 1991b; Nychas and Board 1991; Borch *et al.* 1991). There is no information in the literature concerning the type of beneficial effect, especially as far as it concerns the staphylococci, which may impart a

characteristic flavour through such metabolic activity as lipolysis or proteolysis. An additional and vital aspect of the contribution of these organisms to the final characteristics of the product is related to the reduction of hydrogen peroxide should it be produced by lactobacilli and the development of colour through NO_3 reduction (Cassens *et al.* 1979).

Recently Borch and her co-workers (Borch and Molin 1988, 1989; Borch *et al.* 1991) have studied the taxonomy of psychrotrophic lactic bacteria (eg. *Pediococcus, Lactobacillus, Leuconostoc, Carnobacterium*) as well as their physiology under aerobic or anaerobic conditions with special emphasis on the products of metabolism in batch cultures. The only data available on the metabolic products of *St. carnosus* come from a leaflet of the Crist. Hansen company. This part of our study investigated the effect of the composition of growth media (different broths with or without glucose and oxygen limitation) on the accumulation of metabolic products such as lactate and acetate and on the formation of exoproteins which may contribute to the characteristic attributes of the final product. This approach has been used by the Athens' laboratory and elsewhere over the past few years (Thomas *et al.* 1979; Sedewitz *et al.* 1984; Murphy *et al.*1985; Borch and Molin 1989; Tseng and Montville 1990; Nychas and Board 1991; Nychas *et al.* 1991b)

In order to accelerate growth rate and to accentuate the profile of the physiological products of the test organism, we used an incubation temperature of 37° C instead of 20-25° C which are used during the fermentation of sausages in Europe. High temperature (37° C) is used in sausage fermentation in USA (Bacus 1984; Nychas and Arkoudelos 1990a).

Materials and Methods

The materials and methods are given in detail in the section D, Materials and Methods (pp 46-48). Details of the chemical composition of the media used in this section are given in Table 6.1.

Results

The effect of factors such as oxygen limitation, broth composition and glucose limitation on the different strains of bacteria were analysed with factorial analysis. The results of this analysis are given in Tables 6.2 and 6.3. This analysis (analysis of variance three and four way) was done with the SYSTAT software package and minitab (Penn University, USA). These two Tables list the significant interactions noted in this study. Each of these will be considered in isolation with reference to the appropriate Table containing the detailed results.

Single culture inoculation

Comparison of growth media

The composition of the media used in this study is given in Table 6.1. The statistical analysis (Table 6.2 and 6.3- three way and four way analysis of variance) showed that the type of broth affected significantly the range of the proteins (as demonstrated by the Coomassie blue stain or the Lowry test) as well as the concentration of lactate produced by the test organisms either alone or in combination. Brain Heart Infusion (BHI) contained at the outset much larger amounts of Lowry - positive material than MRS, NZA or NB (Tables 6.4, 6.5, 6.6, 6.7). Furthermore, the total protein (Coomasie blue test) and

Table 6.1 Composition of the media used to study the growth and physicochemical attributes of *Lactobacillus plantarum*, *Staphylococcus carnosus* TM-300 and *Staphylococcus carnosus* DM-20501 in broth cultures

Composition (g l^{-1})	MRS	Nutrient No2	NZA	BHI
Bacteriological peptone (Oxoid)	10			
Lab-Lemco Powder (Oxoid)	8	10		
Yeast Extract (Oxoid)	4		4	
Brain Heart Infusion (Lab M)				17.5
Tryptose (Lab M)				10
NZ Amine (Sheffied Chemical Co. Norwich, N.Y USA)			40	
Peptone		10		
Tween 80	1ml			
Sodium chloride		5		5
Dipotassium hydrogen phosphate	2		1	
Disodium hydrogen phosphate				2.5
Magnesium sulfate $7H_2O$	0.2			
Manganese sulfate $4H_2O$	0.05			
pH	7.0	6.8	7.1	7.0

Table 6.2 The effect of type of media, the amount of glucose added and the oxygen availability on changes in Lowry and Coommasie Blue positive material, as well as on lactic acid production by *Lactobacillus plantarum* and *Staphylococcus carnosus* strains DM-20501 and TM-300 used singly

	Organism								
	O1			O2			O3		
Analysis Factor	1	2	3	1	2	3	1	2	3
Air (A)	ns	***	ns	ns	ns	ns	ns	**	ns
Broth (B)	*	ns	**	***	***	ns	***	**	***
Glucose(C)	ns	ns	ns	ns	*	ns	ns	***	ns
A*B	ns	ns	ns	ns	ns	ns	ns	ns	ns
A*C	ns	**	ns	ns	ns	ns	*	**	ns
B*C	ns	ns	ns	ns	*	ns	ns	*	ns
A*B*C	ns	ns	ns	ns	ns	ns	ns	*	ns

* : The results of two experiments done in duplicate were analysed

O1 : *Lactobacillus plantarum;*
O2 : *Staphyloccus carnosus* DM-20501;
O3 : *Staphylococcus carnosus* TM-300
* : significant at 95 % level
** : significant at 99 % level
***: significant at 99.99% level
ns : not significant
1 : Coomassie Blue positive material; 2: lactic acid;
3 : Lowry positive material

Table 6.3 The effect of type of media, the amount of glucose added, oxygen availability and different combinations of organisms on changes of Lowry and Coommasie Blue positive material, as well as on lactic acid production by *Lactobacillus plantarum* and *Staphylococcus carnosus* strains DM-20501 and TM-300 used in combination*

	Organism								
	O1			O2			O3		
Analysis\ Factor	1	2	3	1	2	3	1	2	3
Air (A)	ns	***	ns	ns	*	ns	ns	ns	ns
Broth (B)	***	*	***	***	***	ns	***	***	***
Glucose(C)	ns	**	ns	ns	**	ns	ns	ns	ns
Strain(D)	ns	**	***	***	***	***	***	**	***
A*B	ns	ns	ns	ns	ns	ns	ns	ns	ns
A*C	ns	***	ns	ns	ns	ns	ns	ns	ns
A*D	ns	ns	ns	ns	ns	ns	ns	**	ns
B*C	ns	ns	ns	ns	*	ns	ns	ns	ns
B*D	ns	ns	***	***	***	ns	**	**	***
C*D	ns	*	ns	ns	*	ns	ns	**	ns
A*B*C	ns	ns	ns	ns	ns	ns	ns	ns	ns
A*B*D	ns	ns	ns	ns	ns	ns	ns	*	ns
A*C*D	ns	*	*	ns	ns	ns	ns	ns	ns
B*C*D	ns	ns	ns	ns	*	ns	ns	ns	ns
A*B*C*D	ns	ns	ns	ns	ns	ns	ns	ns	ns

* : The results of two experiments done in dumplicate were analysed

O1: *Lactobacillus plantarum*;
O2: *Staphylococcus carnosus* DM-20501;
O3: *Staphylococcus carnosus* TM-300

* : significant at 95 % level
** : significant at 99 % level
***: significant at 99.99% level
ns : not significant

1 : Coomassie Blue positive material; 2: lactic acid;
3 : Lowry positive material

Table 6.4 Changes in pH and in the concentration# of extracellular protein&, lactic acid$ and Lowry## positive material during the growth (static or shaking cultures) of *Staphylococcus carnosus* TM-300 in MRS and NB at 37° C

Analysis \ Media*		Time of sampling (h) 0	6	18	24	30	42	48
Static cultures								
pH	- MRS	7.2	7.1	7.3	7.3	7.5	7.6	7.5
	+ MRS	7.2	7.1	5.4	5.4	5.5	5.6	5.6
	- NB	6.4	6.6	7.2	7.2	7.4	7.5	7.4
	+ NB	6.4	6.4	5.2	4.9	4.8	4.6	4.5
Lowry's positive material	- MRS	210	273	229	315	170	272	244
	+ MRS	201	287	326	299	244	189	234
	- NB	278	265	291	295	280	300	269
	+ NB	270	273	292	290	272	242	284
Exoprotein production	- MRS	86.3	129.4	125.4	139.3	129.4	131.9	140.3
	+ MRS	86.0	98.5	103.9	103.8	136.6	132.5	141.3
	- NB	11.3	28.3	nd	20.3	25.4	34.2	29.0
	+ NB	12.2	34.3	nd	23.2	27.8	26.1	20.5
Lactic acid	- MRS	.010	.020	.032	.039	.007	.000	.00
	+ MRS	.010	.022	.162	.168	.062	.136	.06
	- NB	.006	.007	.008	.010	.008	.000	.00
	+ NB	.005	.091	.157	.097	.145	.032	.05
Shaking cultures								
pH changes	- MRS	7.2	7.1	7.5	7.6	8.0	8.5	8.6
	+ MRS	7.2	7.0	6.1	6.4	6.9	7.7	8.0
	- NB	6.4	6.6	7.8	7.9	8.3	8.6	8.5
	+ NB	6.4	6.3	4.9	5.0	5.4	7.0	7.3
Lowry's positive material	- MRS	200	302	330	296	273	234	222
	- NB	270	284	291	296	276	269	297
	+ MRS	205	288	300	293	155	200	147
	+ NB	272	272	290	289	292	226	345
Exoprotein production	- MRS	80.1	90.3	77.6	144.7	145.0	133.6	151.3
	+ MRS	80.4	88.3	91.4	98.4	169.3	162.5	177.5
	- NB	11.5	21.3	nd	30.3	36.8	35.5	34.1
	+ NB	11.5	19.1	nd	22.1	27.6	27.4	31.4
Lactic acid	- MRS	.013	.036	.013	.000	.000	.000	.00
	+ MRS	.013	.063	.038	.000	.000	.000	.00
	- NB	.006	.077	.000	.000	.000	.000	.00
	+ NB	.006	.066	.026	.000	.000	.000	.00

#: Mean of duplicate analyses from one of the two experiments.
$: mg/l; ##: mg/l; &: mg/l; nd: not determined
+: with addition of glucose; -: without addition of glucose

MRS: Mann-Rogosa-Sharp medium; NB: Nutrient Broth No.2 Medium

Table 6.5 Changes in pH and in the concentration[#] of extracellular protein[&], lactic acid[$] and Lowry[##] positive material during the growth (static or shaking cultures) of *Staphylococcus carnosus* TM300 in NZA and BHI under at 37° C.

analysis \ media		Time of sampling (h) 0	6	18	24	30	42	48
Static cultures								
pH	- BHI	7.0	6.9	6.9	6.9	6.9	6.9	6.7
changes	+ BHI	7.0	6.8	6.1	5.8	5.7	5.4	6.0
	- NZA	7.1	6.9	7.2	7.1	7.2	7.3	7.9
	+ NZA	7.1	6.9	6.4	6.1	5.9	6.1	6.7
Lowry's	- BHI	380	429	398	ND	393	375	450
positive	+ BHI	380	333	ND	385	392	410	390
material	- NZA	300	332	ND	328	416	380	410
	+ NZA	300	362	ND	ND	ND	410	350
Exoprotein	- BHI	105	125.7	165.8	ND	119	100.0	ND
production	+ BHI	105	131.0	ND	145.5	ND	163.1	194.3
	- NZA	45	68.5	ND	82.1	ND	90.1	93.0
	+ NZA	45	77.5	ND	120.3	137.1	150.2	ND
Lactic acid	- BHI	.13	.14	.18	ND	.12	.11	ND
	+ BHI	.13	.12	.26	ND	.30	.20	ND
	- NZA	.04	ND	ND	.08	.05	.05	.06
	+ NZA	.04	.09	ND	.41	.48	.46	.40
Shaking cultures								
pH	- BHI	7.0	6.5	6.8	6.8	6.8	6.8	7.3
changes	+ BHI	7.0	6.4	5.0	4.8	4.8	4.8	5.4
	- NZA	7.1	6.9	6.7	7.1	7.1	7.2	7.4
	+ NZA	7.1	6.7	6.2	6.2	6.1	6.1	6.8
Lowry's	- BHI	380	327	ND	328	350	352	378
positive	+ BHI	380	391	ND	409	370	370	383
material	- NZA	300	346	ND	395	338	326	352
	+ NZA	300	417	ND	405	405	367	378
Exoprotein	- BHI	105	158	ND	140	151	120	ND
production	+ BHI	105	120	ND	180	153	161	ND
	- NZA	45	66	ND	87	132	153	ND
	+ NZA	45	ND	ND	165	160	150	195
Lactic acid	- BHI	.13	.19	ND	.09	.11	.09	.06
	+ BHI	.13	.21	ND	.25	.23	.22	.00
	- NZA	.04	ND	ND	.03	.06	.10	.05
	+ NZA	.04	.07	ND	.24	.24	.25	.21

#: Mean of duplicate analyses from one of the two experiments.
$: mg/l; ##: mg/l; &: mg/l; nd: not determined
+: with addition of glucose; -: without addition of glucose

NZA: N-Z Amine medium ; BHI: Brain Heart Infusion medium

Table 6.6 Changes in pH and in the concentration[#] of extracellular protein[&], lactic acid[$] and Lowry[##] positive material during the growth (static or shaking cultures) of *Staphylococcus carnosus* DM-20501 in MRS and NB under at 37° C.

analysis \ media		Time of sampling (h) 0	6	18	24	30	42	48
Static cultures								
pH changes	- MRS	7.0	7.0	7.0	7.0	7.0	7.1	7.1
	+ MRS	7.0	7.0	5.4	5.4	5.8	6.8	6.8
	- NB	6.8	6.7	7.0	7.0	7.1	7.3	7.3
	+ NB	6.8	6.5	5.0	4.7	4.7	4.6	4.6
Lowry's positive material	- MRS	357	350	183	308	282	268	220
	+ MRS	355	337	258	316	314	217	208
	- NB	319	311	219	265	325	235	221
	+ NB	312	300	210	274	279	239	186
Exoprotein production	- MRS	85.5	121.2	134.6	120.2	136.4	164.2	136.0
	+ MRS	84.2	88.2	120.1	131.1	103.1	114.1	58.0
	- NB	11.3	32.3	39.3	53.4	69.6	42.5	26.5
	+ NB	11.3	24.5	47.3	38.5	50.0	44.3	37.5
Lactic acid	- MRS	.082	.100	.153	.156	.145	.162	.140
	+ MRS	.081	.090	.399	.446	.286	.415	.300
	- NB	.006	.072	.136	.153	.110	.097	.095
	+ NB	.006	.096	.327	.400	.355	.328	.318
Shaking cultures								
pH changes	- MRS	7.0	7.1	7.1	7.1	7.3	7.8	7.9
	+ MRS	7.0	7.0	5.4	5.3	5.5	6.2	6.6
	- NB	6.8	6.7	7.4	7.4	7.6	8.1	8.3
	+ NB	6.8	6.6	4.6	4.5	4.7	5.2	5.8
Lowry's positive material	- MRS	356	230	159	300	327	195	300
	+ MRS	356	350	258	322	246	241	180
	- NB	318	299	218	227	302	230	238
	+ NB	310	322	171	300	286	225	200
Exoprotein production	- MRS	78.9	97.7	110.1	151.3	157.2	162.3	162.2
	+ MRS	78.9	104.2	98.3	142.0	121.1	137.6	144.9
	- NB	11.5	37.3	42.6	54.8	56.4	79.4	44.9
	+ NB	11.5	16.7	62.2	68.5	36.5	33.1	24.5
Lactic acid	- MRS	.071	.082	.100	.116	.092	.000	.00
	+ MRS	.071	.095	.325	.333	.175	.005	.00
	- NB	.006	.010	.062	.048	.000	.000	.00
	+ NB	.006	.070	.313	.158	.136	.000	.00

#: Mean of duplicate analyses from one of the two experiments.
$: mg/l; ##: mg/l; &: mg/l; nd: not determined
+: with addition of glucose; -: without addition of glucose

MRS: Mann-Rogosa-Sharp medium; NB: Nutrient Broth No.2 Medium

Table 6.7 Changes in pH and in the concentration# of extracellular protein&, lactic acid$ and Lowry## positive material during the growth (static or shaking cultures) of *Lactobacillus plantarum* in MRS and NB under at 37° C

analysis \ media		Time of sampling h 0	6	18	24	42	48
Static cultures							
pH changes	- MRS	7.1	7.1	7.1	7.0	7.0	7.1
	+ MRS	7.1	7.1	7.1	7.0	7.1	7.1
	- NB	6.6	6.6	6.6	6.5	7.2	7.2
	+ NB	6.6	6.4	6.5	6.2	6.2	5.8
Lowry's positive material	- MRS	250	236	270	270	313	354
	+ MRS	250	240	170	300	255	293
	- NB	275	286	275	304	257	281
	+ NB	275	297	318	317	234	270
Exoprotein production	- MRS	100	90	61	84	110	172
	+ MRS	100	74	92	115	130	200
	- NB	30	30	33	44	nd	68
	+ NB	30	43	nd	54	nd	72
Lactic acid	- MRS	.03	.10	.10	.10	.12	.09
	+ MRS	.03	.07	.12	.15	.25	.35
	- NB	.03	.09	.10	.12	.16	.14
	+ NB	.03	.06	.06	.08	.13	.21
Shaking cultures							
pH changes	- MRS	7.1	7.1	7.1	7.1	7.1	7.1
	+ MRS	7.1	7.1	7.1	7.0	6.0	6.5
	- NB	6.6	6.7	7.0	7.0	7.1	7.1
	+ NB	6.6	6.5	6.5	6.3	4.7	4.7
Lowry's positive material	- MRS	250	272	294	272	290	290
	+ MRS	250	230	214	266	256	257
	- NB	275	255	263	295	275	270
	+ NB	275	252	270	310	290	263
Exoprotein production	- MRS	100	134	122	154	111	161
	+ MRS	100	92	92	105	115	163
	- NB	30	39	nd	40	37	61
	+ NB	30	27	nd	42	57	68
Lactic acid	- MRS	.03	.06	.103	.065	.100	0
	+ MRS	.03	.11	.07	.040	.004	0
	- NB	.03	.07	.08	.090	.090	.1
	+ NB	.03	.10	.15	.050	.006	0

#: Mean of duplicate analyses from one of the two experiments.
$: mg/l; &: mg/l; nd: not determined
+: with addition of glucose; -: without addition of glucose

MRS: Mann-Rogosa-Sharp medium; NB: Nutrient Broth No.2 Medium

lactic acid contents were greater in BHI at time zero than in Nutrient Broth, NZA or MRS media (Table 6.4, 6.5, 6.6, 6.7).

In the case of both strains of *St. carnosus* (TM-300 and DM-20501) as well as *L. plantarum*, the extent of O.D. (550nm) in MRS medium (Tables 6.8, 6.9, 6.10) in the vast majority of cases was higher than that in the other media used in this study under static or shaking conditions. The exception was *Staph. carnosus* TM-300 grown in BHI without additional glucose or shaking. This difference affected, as one would expect, the final size of the bacterial counts in these media. Indeed the size of bacterial counts of these organisms, enumerated on MRS medium, was *ca.* 0.5-1 log higher than those in NB, NZA and BHI in samples subjected to similar conditions (Table 6.11).

Effect of glucose

The three-way statistical analysis (Table 6.2 and 6.3) revealed that the addition of glucose did not affect the extent of exoprotein production or the final concentration of Lowry- positive material whereas lactate production was influenced on two occasions (with *St. carnosus* TM-300 and DM-20501 under shaking conditions).

The addition of glucose to a medium stimulated the growth {as expressed by Optical Density (OD) at 550 nm} of both strains of *Staph. carnosus* and *Lact. plantarum* both in static or shaking conditions (Tables 6.8-6.10). In all cases with static and shaking samples, glucose addition to a medium inoculated with *St. carnosus* resulted, as judged by O.D. measurement, in a diminished lag phase and more extensive growth during the logarithimic growth phase. The actual cell densities as judged by viable counts at the end of 48h incubation

Table 6.8 Growth[#] (O. D. at 550 nm) of *Staphylococcus carnosus* TM-300 in MRS, NB, BHI and NZA media with or without glucose in static or shaking cultures at 37° C

		Time of Sampling (Hours)						
Medium	G/O	0	6	18	24	30	42	48
MRS	-/-	.010	.038	.05	.065	.07	.08	.104
MRS	+/-	.010	.038	.14	.163	.17	.18	.19
MRS	-/+	.010	.043	.31	.440	.54	.61	.69
MRS	+/+	.010	.040	.31	.520	.49	.67	.80
NB	-/-	.010	.010	.002	.036	.039	.045	.060
NB	+/-	.010	.020	.067	.090	.100	.112	.125
NB	-/+	.010	.018	.364	.422	.437	.460	.500
NB	+/+	.010	.022	.328	.422	.450	.470	.550
BHI	-/-	.010	.020	.040	.045	.040	.075	.111
BHI	+/-	.010	.030	.140	.160	.150	.155	.150
BHI	-/+	.010	.040	.070	.110	.100	.120	.140
BHI	+/+	.010	.130	.250	.250	.240	.240	.240
NZA	-/-	.010	.010	.070	.075	.080	.075	.07
NZA	+/-	.010	.010	.110	.160	.160	.165	.160
NZA	-/+	.010	.010	.140	.180	.190	.190	.190
NZA	+/+	.010	.100	.200	.210	.200	.200	.220

G: Glucose, -, without; +, with
O: Oxygen, -, static; +, shaken

NZA: N-Z amine medium; MRS: Man-Rogosa-Sharpe medium;
NB: Nutrient broth medium; BHI: Brain Heart Infusion medium

#: Mean of duplicate analyses from one of the two experiments.

Table 6:9 Growth# (O. D. at 550 nm) of *Staphylococcus carnosus* DM-20501 in MRS, NB and NZA media with or without glucose in static or shaking cultures at 37° C

Medium	G/O	Time of Sampling (Hours) 0	6	18	24	30	42	48
MRS	-/-	.020	.030	.06	.065	.08	.075	.100
MRS	+/-	.020	.040	.11	.115	.105	.115	.105
MRS	-/+	.020	.020	.025	.183	.280	.500	.668
MRS	+/+	.020	.040	.200	.250	.283	.540	.690
NB	-/-	.010	.010	.030	.033	.045	.046	.055
NB	+/-	.010	.010	.080	.080	.080	.090	.080
NB	-/+	.010	.012	.015	.200	.255	.380	.400
NB	+/+	.010	.010	.015	.160	.220	.350	.420

G: Glucose, -, without; +, with
O: Oxygen, -, static; +, shaking

MRS: Man-Rogosa-Sharpe medium; NB: Nutrient broth medium

#: Mean of duplicate analyses from one of the two experiments.

Table 6.10 Growth[#] (O. D. at 550 nm) of *Lactobacillus plantarum* in MRS and NB media with or without glucose in static or shaking cultures at 37° C

Medium	G/O	Time of Sampling (Hours) 0	6	18	24	42	48	72
MRS	-/-	.020	.041	.030	.033	.045	.065	.070
MRS	+/-	.020	.023	.040	.045	.050	.075	.080
MRS	-/+	.020	.020	.021	.050	.060	.100	.120
MRS	+/+	.020	.020	.050	.080	.469	.629	.650
NB	-/-	.010	.011	.030	.033	.045	.055	.060
NB	+/-	.010	.023	.040	.045	.045	.065	.080
NB	-/+	.010	.022	.050	.060	.090	.100	.180
NB	+/+	.010	.010	.050	.100	.255	.270	.290

G: Glucose, -, without; +, with
O: Oxygen, -, static; +, shaken

MRS: Mann-Rogosa-Sharpe medium; NB: Nutrient broth medium

#: Mean of duplicate analyses from one of the two experiments.

Table 6.11. Final growth of *St. carnosus* strains TM300, DM-20501 and *Lact. plantarum* either alone or in combination, in various media with or without addition of glucose and/or oxygen limitation at 37° C

Media	G/O	Organism (log_{10})				
		St. carnosus TM-300	*St.carnosus* DM-20501	*L.plantarum**	*St.carnosus* + *L. plantarum*	
		(5.6)[&]	(4.6)	(2.8)	(5.5)	(4.3)
MRS	-/-	8.9	8.4	6.6	8.0	8.0
MRS	+/-	9.0	8.5	6.7	8.5	8.5
MRS	-/+	10.2	9.0	9.0	9.0	9.3
MRS	+/+	9.2	9.1	9.3	8.8	9.4
		(5.6)	(4.6)	(2.8)	(5.5)	(4.3)
NB	-/-	8.1	7.9	7.0	8.0	8.2
NB	+/-	8.2	8.3	7.8	8.4	8.5
NB	-/+	9.2	9.1	6.7	8.8	9.0
NB	+/+	9.0	8.8	7.3	8.8	9.2
		(5.5)			(4.0)	(4.2)
NZA	-/-	8.4	nd	nd	8.4	8.3
NZA	+/-	8.4			9.2	8.4
NZA	-/+	8.0			-	-
NZA	+/+	8.3			-	-
		(5.5)			(4.0)	(4.2)
BHI	-/-	8.7	nd	nd	-	-
BHI	+/-	8.0			-	-
BHI	-/+	8.2			9.0	9.5
BHI	+/+	8.2			9.4	9.5

&: Numbers in parenthesis initial size of inoculum in media
G: Glucose, -, without; +, with
O: Oxygen, -, static; +, shaken

NZA: N-Z amine medium; BHI: Brain heart Infusion medium;
NB: Nutrient broth medium; MRS: Mann-Rogosa-Sharpe medium

* : Growth after 72h

nd: not determined

Staphylococcus carnosus was isolated on MASA and *Lactobacillus plantarum* on MRS

(Table 6.11), however, did not differ significantly. Similar results were found with *L. plantarum*, although the lag period was more extensive and $\log_{10}8$ cfu/ml was obtained by 72 h incubation (Table 6.11).

The rates of glucose utilisation by *L. plantarum* and *St. carnosus* TM-300 and DM-20501 in different media are shown in Table 6.12. It was found with the latter two strains that, with the exception of NB without additional glucose, the consumption of this carbohydrate was > 90% by 24h of incubation. This rapid utilization of glucose accentuated the acid drift (Tables 6.3-6.4) due to an increased production of lactic acid. The pH drop in NB with glucose was much greater than that in BHI, NZA or MRS. There was an alkaline drift in the various media with glucose. This drift was more pronounced in samples under shaking conditions (Tables 6.4-6.7).

Products of metabolism

Exoprotein

The concentration of exoproteins was affected by the type of broth. Although glucose and oxygen limitation did not influence actual exoprotein production, the final 'absolute' values of exoprotein were generally higher in samples supplemented with glucose and having access to oxygen *vis a vis* samples without glucose or incubated statically. Indeed with both strains, TM-300 and DM-20501, of *St. carnosus* and *L. plantarum*, it was found that the rate of exoprotein production was higher in MRS than in NB with or without glucose under both conditions (static or shaking) (Tables 6.4, 6.6, and 6.7). Similar trends were evident with NZA for *St.carnosus* TM-300 in BHI medium (Table 6.5). This could be attributed to the composition of NZA medium and to the fact that NB and NZA contain readily available nitrogen sources (peptides,

Table 6.12 Percentage[#] of glucose consumption in MRS, NB and NZA media by *Staphylococcus carnosus* DM-20501 *Staphylococcus carnosus* TM-300 and *Lactobacillus plantarum* under static and shaking cultures at 37° C

Medium	O	Time of Sampling (Hours) 0	6	18	24	30	42	48
***Staphylococcus carnosus* DM-20501**								
MRS	-	0	12.5	96.5	97	97	99	99
MRS	+	0	13	98.5	99	99	99	99
NB	-	0	15	52.5	65	71.5	83.6	90
NB	+	0	13	94	95	95	96	97
***Staphylococcus carnosus* TM-300**								
MRS	-	0	32	99.5	99.5	99.5	99.9	100
MRS	+	0	24	99	99.5	99.6	99.9	100
NB	-	0	1	50	60	70	82	99
NB	+	0	6	93.5	94	95	95	100
BHI	-	0	10	-	54	58	66	91
BHI	+	0	12	-	96	99	99.5	99.7
NZA	-	0	5	-	90	95	95.5	98.3
NZA	+	0	26.8	-	97.4	99	99.3	100
Lactobacillus plantarum								
MRS	-	0	21.5	25	27	30	34	35
MRS	+	0	7.5	8	8.5	100	100	100
NB	-	0	5	5	5	25	30	35
NB	+	0	6	13.5	30	91	92	99

#: Mean of duplicate analyses from one of the two experiments.
O: Oxygen, -, static; +, shaking

NZA: N-Z amine medium; MRS: Mann-Rogosa-Sharpe medium;
NB : Nutrient broth medium; BHI: Brain Heart Infusion medium

amino acids). When the protein profiles of cultures grown in NB with or without glucose were compared, differences were observed in the range of proteins produced (Figure 6.1 and 6.2).

Lactic and acetic acid

Similar results to those discussed above were observed in respect of the production of lactate. According to three way analysis of variance, the addition of glucose did not affect final lactic acid production by shaking cultures of *L. plantarum* (Table 6.2), but it did so with *St. carnosus* strains TM-300 and DM-20501. Lactate production was influenced (90% significance) by oxygen availability and the type of broth (Table 6.2) in almost all cases. In samples with oxygen limitation (static cultures), lactic acid production occurred throughout the incubation period when glucose was present (Tables 6.4, 6.5, 6.6 and 6.7). In shaken samples the maximum yield of lactic occurred mainly during the first part of the incubation period but the initial increase of lactate was followed by a marked loss (Tables 6.4,6.5,6.6 and 6.7). This pattern in lactate production, an increase followed by a loss, was found in MRS and NB inoculated with *L. plantarum* and *St. carnosus* TM-300 (Table 6.4 and 6.7), with strain DM-20501 (Table 6.6) and in NZA and BHI inoculated with *St. carnosus* TM-300 (Table 6.5). The extent of lactate production in NB tended to be higher than in MRS which contained initially larger amounts of lactate than did NB (Tables 6.4, 6.6 and 6.7). Similarly the absolute value of the rate of lactate production in NZA samples inoculated with *St. carnosus* TM-300 was higher than that found in BHI which also contained a high initial concentration of lactate derived from the ingredients (Table 6.5).

With acetic acid production (Table 6.13), it was found that the addition of glucose influenced significantly the

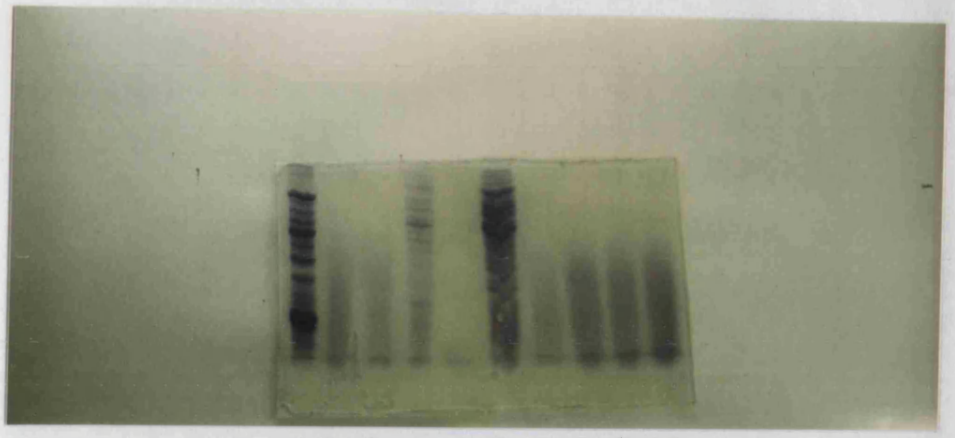


Fig. 6.1 SDS-Polyacrylamide gel of extracted proteins from broth cultures of *Lactobacillus plantarum* (LAB), *Staphylococcus carnosus* TM-300 (TM) and *Staphylococcus carnosus* DM-20501 (DM) after growth for 48 hours at 37° C.

Gels 1 to 10 represent:

Gel	Organism	Medium	Conditions	Time of growth
1	Standard proteins			
2	TM	Nutrient broth + glucose 0.2%	static	48h
3	DM	MRS Broth + glucose 0.2%	shaking	0h
4	DM	MRS Broth + glucose 0.2%	shaking	48h
5	LAB	NZA Broth + glucose 0.2%	shaking	0h
6	LAB	NZA Broth + glucose 0.2%	shaking	48h
7	LAB	Nutrient Broth	static	0h
8	LAB	Nutrient Broth	static	48h
9	LAB	Nutrient Broth + glucose 0.2%	static	0h
10	LAB	Nutrient Broth + glucose 0.2%	static	48h

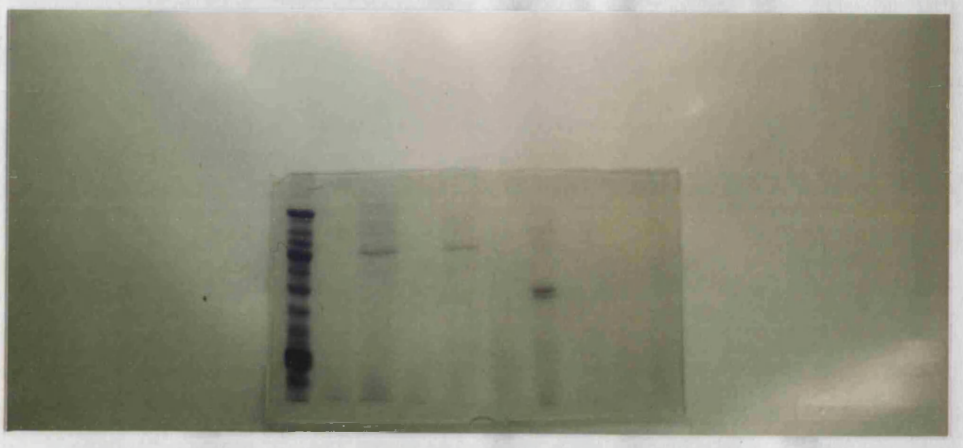
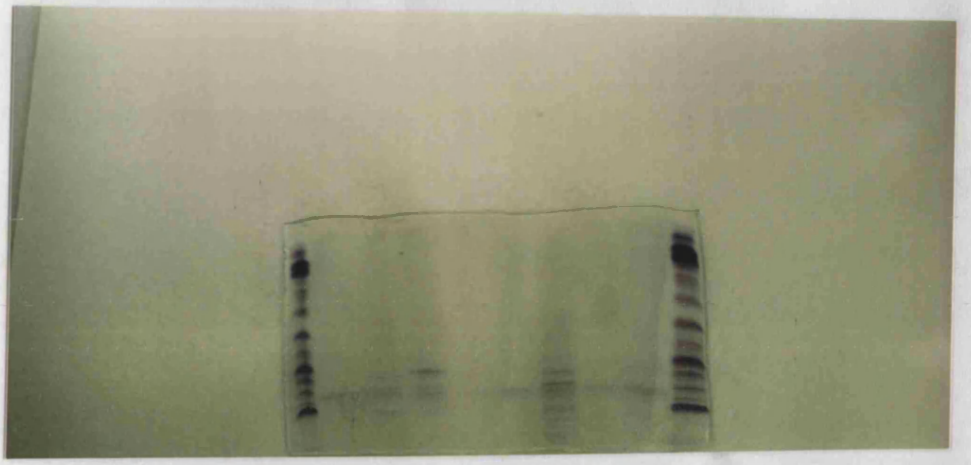


Fig. 6.2 SDS-Polyacrylamide gel of extracted proteins from broth cultures of *Lactobacillus plantarum* (LAB), or *St. carnosus* DM-20501 (DM) after growth for 48 hours at 37° C.

Gels 1 to 10 represent:

Gel	Organism	Medium	Conditions	Time of growth
1	Standard proteins			
2	DM	MRS broth	shaking	0h
3	DM	MRS broth	shaking	48h
4	DM	Nutrient broth	shaking	0h
5	DM	Nutrient broth	shaking	48h
6	LAB	MRS broth + glucose 0.2%	shaking	0h
7	LAB	MRS broth	shaking	48h
8.	LAB	MRS broth	static	0h
9	LAB	MRS broth	static	48h
10	DM	Nutrient broth +glucose 0.2%	static	0h



1 2 3 4 5 6 7 8 9 10

Fig. 6.3 SDS-Polyacrylamide gel of extracted proteins from broth cultures of *Lactobacillus plantarum* (LAB) or *St. carnosus* TM-300 (TM) after growth for 48 hours at 37° C.

Gels 1 to 10 represent:

Gel	Organism	Medium	Conditions	Time of growth
1	Standard proteins			
2	TM	BHI broth	static	0h
3	TM	BHI broth	static	48h
4	LAB	BHI broth + glucose 0.2%	shaking	0h
5	LAB	BHI broth + glucose 0.2%	shaking	48h
6	TM	BHI broth	shaking	0h
7	TM	BHI broth	shaking	48h
8	LAB	BHI broth	static	0h
9	LAB	BHI broth	static	48h
10	Standard proteins			

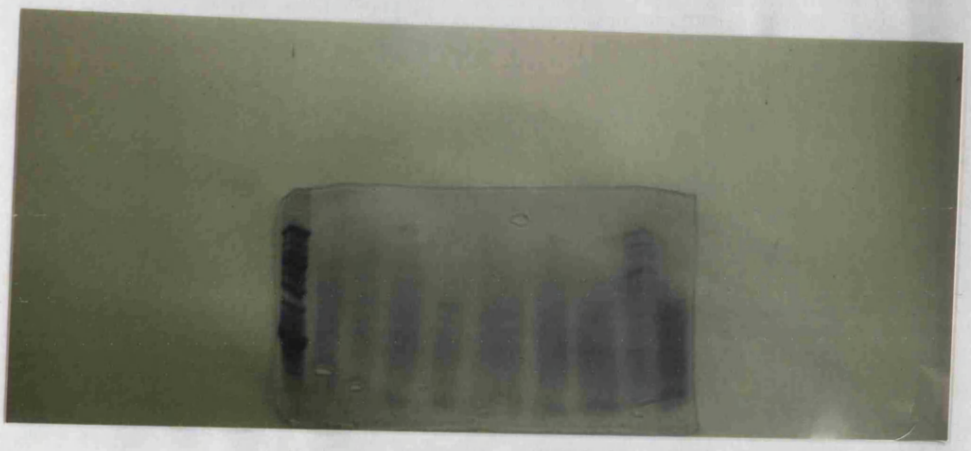


Fig. 6.4 SDS-Polyacrylamide gel of extracted proteins from broth cultures of *Lactobacillus plantarum* (LAB), *St. carnosus* TM-300 (TM) or *St. carnosus* DM-20501 (DM) after growth for 48 hours at 37° C.

Gels 1 to 10 represent:

Gel	Organism	Medium	Conditions	Time of growth
1	Standard proteins			
2	DM	Nutrient broth	static	0h
3	DM	Nutrient broth	static	48h
4	LAB	Nutrient broth	shaking	0h
5	LAB	Nutrient broth	shaking	48h
6	DM	MRS broth + glucose 0.2%	static	0h
7	DM	MRS broth + glucose 0.2%	static	48h
8.	TM	MRS broth + glucose o.2%	shaking	0h
9	TM	MRS broth + glucose 0.2%	shaking	48h
10	TM	Nutrient broth +glucose 0.2%	static	0h

Table 6.13 Initial and final concentration of acetate (mg l^{-1}) in Nutrient Broth inoculated with *Lactobacillus plantarum* or *Staphylococcus carnosus* either alone or in combination under static culture at 37° C.

Glucose added	time of sampling	*Lact.plantarum*	*St.carnosus*	*Lact.plantarum + St. carnosus*
no	0h	0.34	0.34	0.34
no	48h	0.50	1.06	2.05
yes	0h	0.34	0.34	0.34
yes	48h	2.00	1.92	3.45

#: Mean of duplicate analyses from one of the two experiments.

final concentration of this acid in NB in static cultures of *L. plantarum* or *St. carnosus*. The prohibitive cost of this test was the main reason why I did not analyse systematically all the experiments. The contribution of this acid,however, is evident in Figs 6.5, 6.6 and 6.7. Indeed the gas-liquid chromatographic profiles at different stages of growth in broth cultures inoculated with *L. plantarum* or *St. carnosus* show clearly an increase in the concentration of this acid throughout the incubation period.

Lowry-positive material

The concentration of water soluble compounds tested with the Lowry reagent fluctuated during incubation. Even so there was generally a slight increase in concentration during incubation with *L. plantarum* (Table 6.4) in media with or without additional glucose both in static and shaking cultures at 37° C. Similar results were obtained with *St. carnosus* strain TM-300 in MRS, NB, BHI and NZA (Tables 6.4 and 6.5). With strain DM-20501, the Lowry-positive material in MRS and NB diminished with time (Table 6.6).

Effect of aeration

Staphylococcus carnosus strains TM-300 and DM-20501 as well as *L. plantarum* were grown aerobically (in shaking bottles) and under reduced oxygen condition (in static bottles) for 48h at 37° C in MRS, NB with or without additional glucose (Tables 6.4, 6.6 and 6.7) NZA or BHI again with or without glucose added (Table 6.5). Oxygen availability did not affect the concentrations of total exoprotein or the Lowry-positive material formed during incubation (Table 6.2 and 6.3). On the other hand the presence of oxygen reduced the concentration of lactate (Tables 6.4-6.7). When samples were analysed by SDS-PAGE,

Fig. 6.5 The acetic acid production by *Lactobacillus plantarum* in MRS medium after 48h of incubation at 37° C

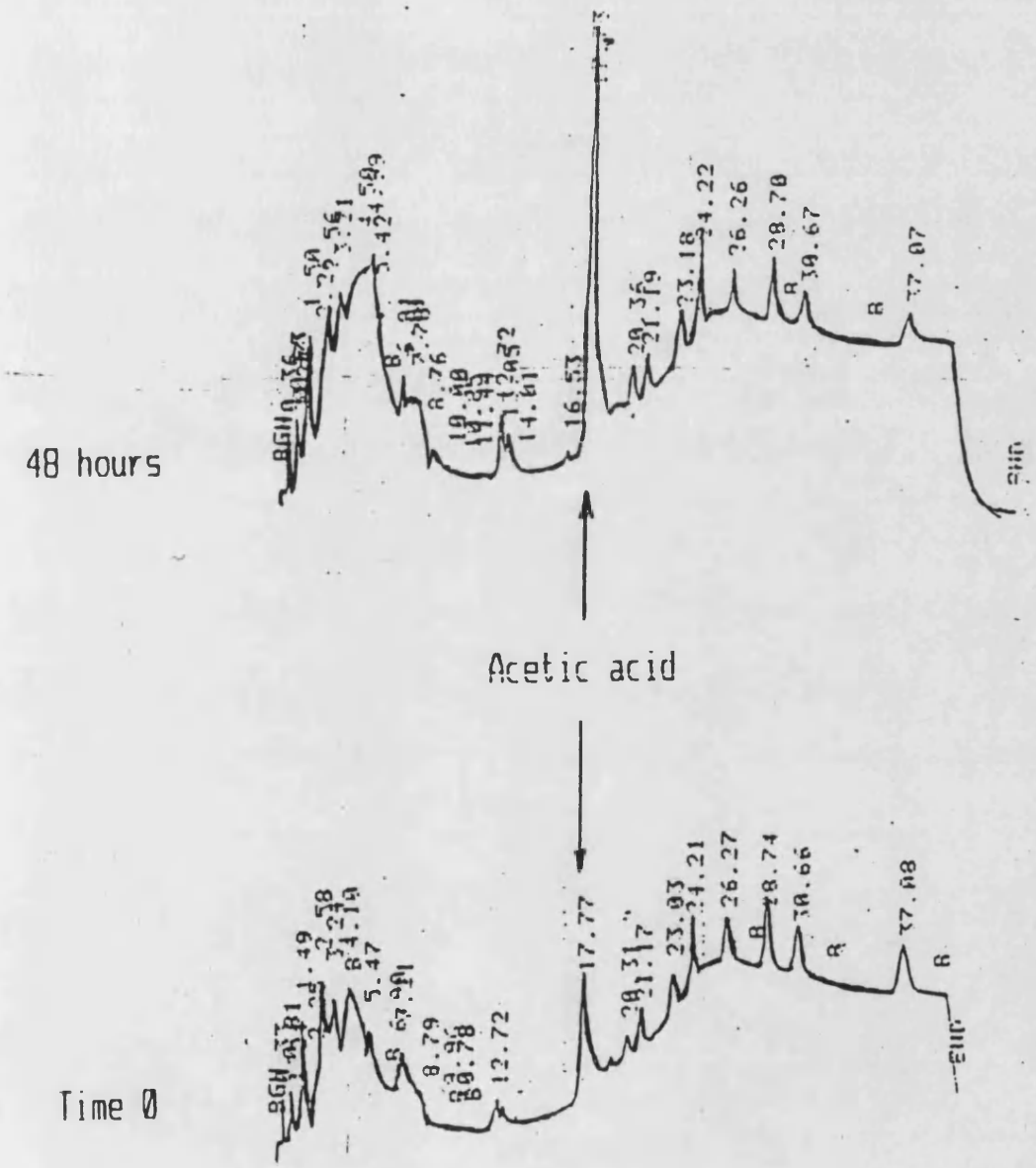


Fig. 6.6 The acetic acid production by *Lactobacillus plantarum* in Nutrient broth No 2 after 48h of incubation at 37° C

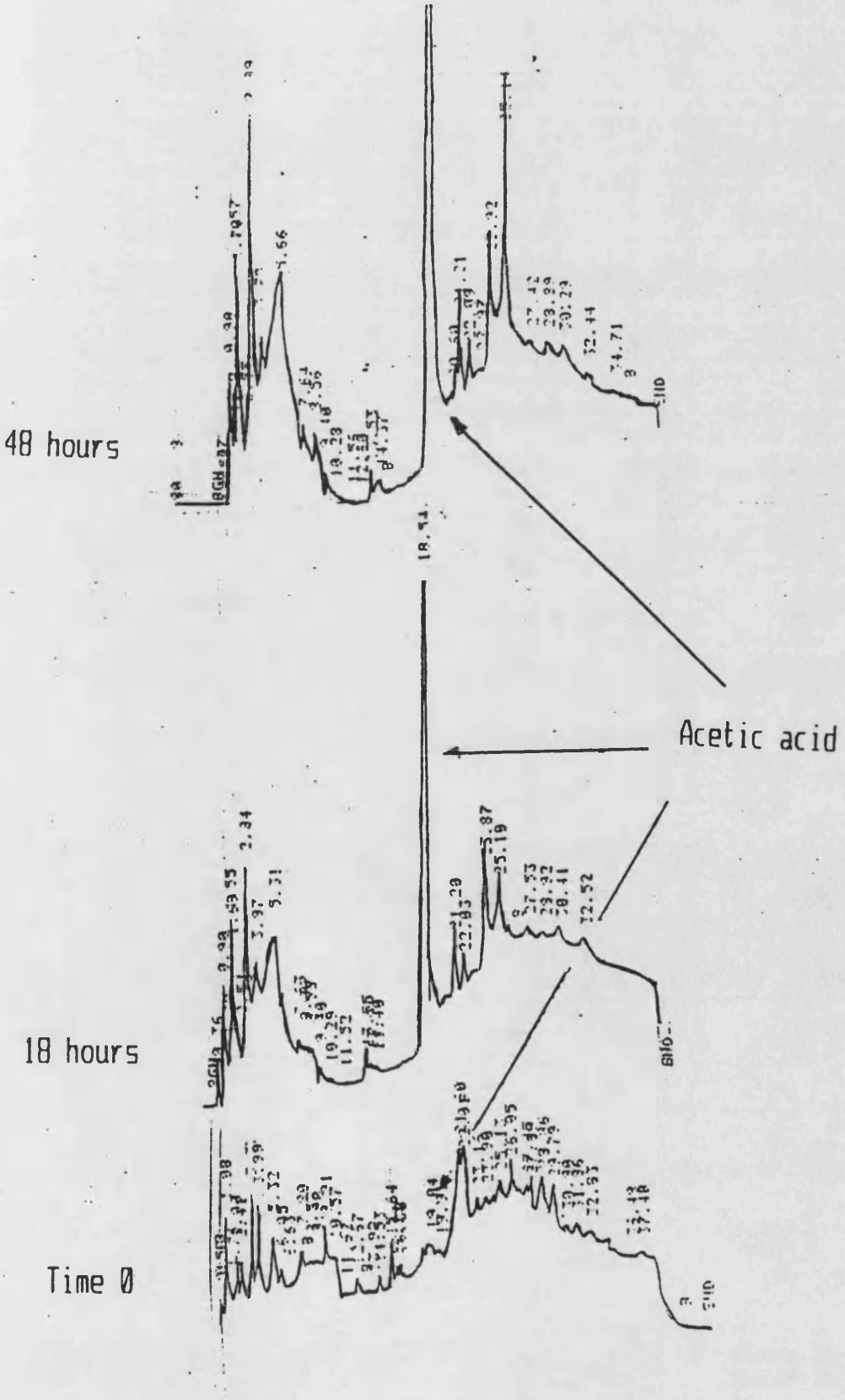
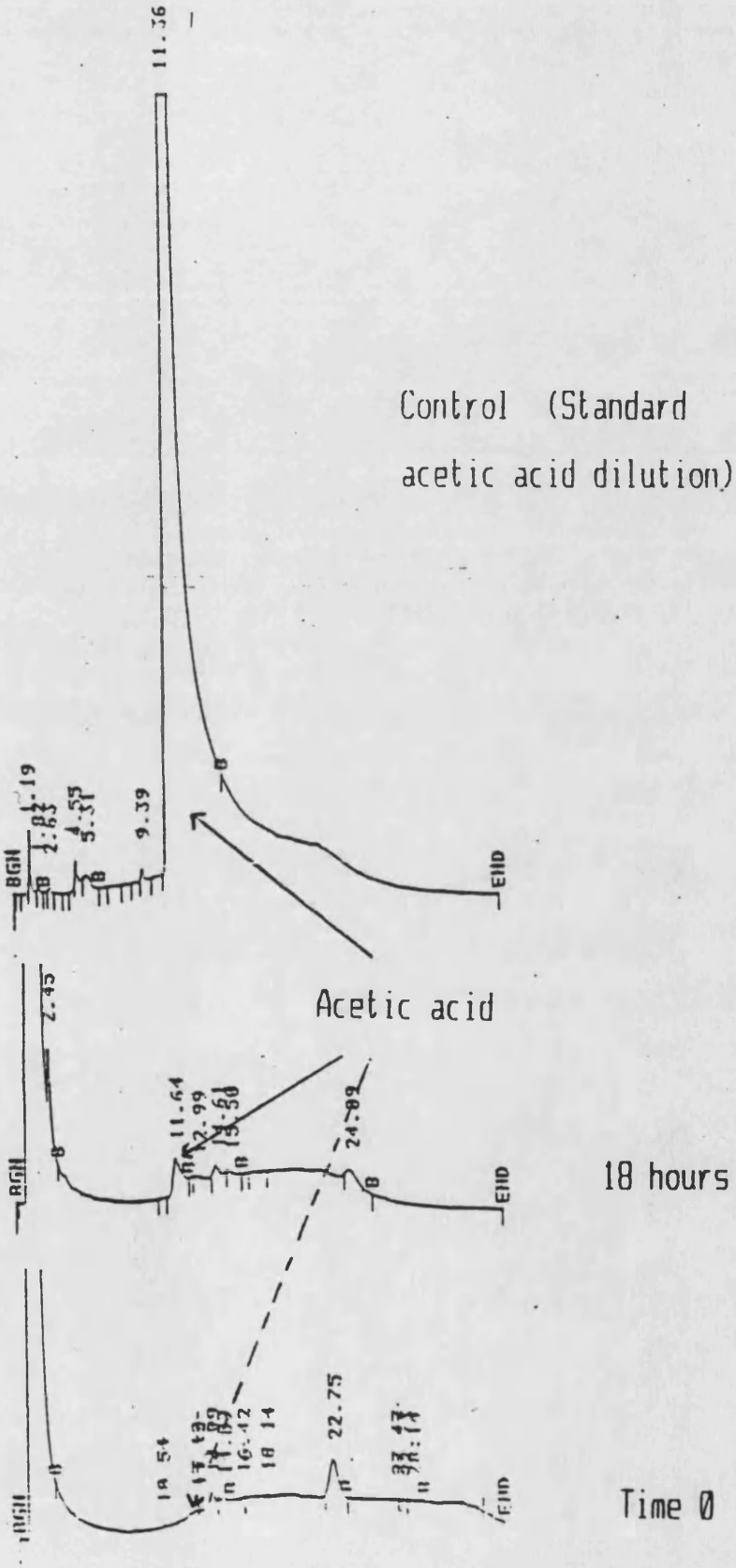


Fig. 6.7 The acetic acid production by *Staphylococcus carnosus* in Nutrient broth No 2 after 18h of incubation at 37° C



different patterns of extracellular proteins in the static and shaken cultures as well as in the two growth media were evident (Figs. 6.1, 6.2, 6.3 and 6.4).

Combined inoculations

The results obtained from the inoculation of MRS,NB, NZA or BHI with both *St. carnosus* and *L. plantarum* are shown in Tables 6.14, 6.15, 6.16, 6.17.

Comparison of the growth media

Tables 6.11 and 6.14 show the size of the final population (cells /ml medium) as well as their absorbance respectively. Although the growth (as expressed by O.D. at 550nm) of these 2 organisms in combination always appeared to be larger in MRS than that observed in NB medium, the number of the cells in these two media did not differ significantly with either static or shaking incubations. It was noted as well that the O.D. obtained with samples inoculated with both bacteria was lower than that found in samples inoculated with *St. carnosus* alone (Tables 6.8,6.9, 6.14).

Effect of glucose

The extent of O.D. of cultures of both organisms in broths(MRS,NB)-(Table 6.14) was increased by the addition of glucose to the medium. Glucose did not, however, significantly influence the number of cells (Table 6.11). It needs to be stressed that when both organisms were inoculated in a medium, the rate of glucose consumption was greater than in samples inoculated with *L. plantarum* or *St. carnosus* alone (Tables 6.12 and 6.17). Indeed with the exception of NB medium, the extent of glucose consumption was 100% during the first 18h of incubation, cf >24h in samples inoculated with a single organism

Table 6.14 Growth# (O. D. at 550 nm) of *Staphylococcus carnosus* and *Lactobacillus plantarum* in MRS and NB media with or without glucose in static or shaking cultures at 37° C

Medium	G/O	Time of Sampling (Hours) 0	6	18	24	30	42	48
MRS	-/-	.010	.010	.030	.040	.060	.070	.080
MRS	+/-	.010	.010	.050	.105	.120	.130	.140
MRS	-/+	.010	.015	.100	.200	.350	.500	.530
MRS	+/+	.010	.020	.120	.215	.400	.510	.600
NB	-/-	.010	.010	.010	.035	.045	.050	.060
NB	+/-	.010	.010	.040	.067	.076	.085	.100
NB	-/+	.010	.012	.060	.200	.237	.369	.340
NB	+/+	.010	.010	.110	.190	.218	.320	.381

G: Glucose, -, without; +, with
O: Oxygen, -, static; +, shaken

MRS: Mann-Rogosa-Sharpe medium; NB: Nutrient broth medium

#: Mean of duplicate analyses from one of the two experiments.

Table 6.15 Changes in pH and in the concentration[#] of extracellular protein[&] and lactic acid[$], during the growth of *Lactobacillus plantarum* and *Staphylococcus carnosus* TM-300 in MRS and NB under static and shaking cultures at 37° C

analysis \ media		Time of sampling h 0	6	18	24	30	42	48
Static cultures								
pH changes	- MRS	7.1	7.1	7.1	7.1	7.2	7.3	7.2
	+ MRS	7.1	7.1	6.0	5.1	5.3	5.5	5.6
	- NB	6.6	6.5	6.8	6.8	8.8	7.1	7.1
	+ NB	6.6	6.5	5.5	5.1	5.0	4.9	4.8
Exoprotein production	- MRS	250.8	144.8	166.7	90.7	53.1	86.1	nd
	+ MRS	250.8	302.8	360.0	397.0	377.8	376.0	
	- NB	99.5	54.9	52.2	43.0	nd	38.5	nd
	+ NB	99.5	154.1	152.9	171.9	131.4	158.4	nd
Lactic acid	- MRS	.080	.030	.030	.016	.029	.081	.103
	+ MRS				nd			
	- NB	.060	.120	.090	.080	.135	.070	.090
	+ NB	.060	.100	.21	.22	.30	.33	.35
Shaking cultures								
pH changes	- MRS	7.1	7.1	7.1	7.2	7.7	8.2	8.5
	+ MRS	7.1	7.1	5.2	5.4	6.0	6.9	7.3
	- NB	6.6	6.5	7.0	7.2	6.6	8.4	8.5
	+ NB	6.6	4.5	4.5	4.6	4.7	4.8	4.7
Exoprotein production	- MRS	254.0	205.0	202.5	162.5	156.0	114.0	nd
	+ MRS	253.0	277.7	256.0	284.0	274.0	284.0	nd
	- NB	99.1	38.6	55.0	74	59.1	42.3	nd
	+ NB	99.1	129.4	179.1	155.5	166.6	205.4	nd
Lactic acid	- MRS	.08	.018	.007	.001	.002	.003	.002
	+ MRS	.08	.16	.44	.33	.03	.007	.002
	- NB				nd			
	+ NB				nd			

#: Mean of duplicate analyses from one of the two experiments
$: mg/l; ##: mg/l; &: mg/l; nd: not determined
+: with addition of glucose; -: without addition of glucose

MRS: Mann-Rogosa-Sharp medium; NB: Nutrient Broth No.2 Medium

Table 6.16 Changes in pH and in the concentration[#] of extracellular protein[&], lactic acid[$] and Lowry's[##] positive material during the growth of *Lactobacillus plantarum* and *Staphylococcus carnosus* TM300 in NZA and BHI under static and shaking cultures at 37° C

Analysis \	Media	Time of sampling (h) 0	12	18	36	42
Static cultures						
pH changes	- BHI	6.9	6.7	6.8	6.8	6.9
	- NZA	7.0	7.1	7.1	7.2	7.2
Lowry's positive material	- BHI	430	189	164	102	137
	- NZA	250	190	185	282	472
Exoprotein production	- BHI	250	245	242	263	260
	- NZA	55	96	104	145	148
Lactic acid	- BHI	.25	.25	.25	.22	.22
	- NZA	.02	.09	.21	.18	.15
Shaking cultures						
pH changes	+ BHI	6.9	6.7	5.9	6.5	6.7
	+ NZA	7.0	6.4	6.5	6.8	7.0
Lowry's positive material	+ BHI	430	190	186	245	471
	+ NZA	250	212	497	280	222
Exoprotein production	+ BHI	250	306	310	307	320
	+ NZA	55	205	370	320	350
Lactic acid	+ BHI	.25	.37	.25	.006	.002
	+ NZA	.02	.38	.37	.02	.000

#: Mean of duplicate analyses from one of the two experiments.
$: mg/l; ##: mg/l; &: mg/l; nd: not determined
+: with addition of glucose; -: without addition of glucose

NZA: N-Z Amine medium ; BHI: Brain Heart Infusion medium

Table 6.17 Percentage# of glucose consumption in MRS, NB and NZA media by *Staphylococcus carnosus* together with *Lactobacillus plantarum* under static and shaking cultues at 37° C

		Time of sampling (hours)						
Medium	O	0	6	18	24	30	42	48
MRS	-	0	12	100	100	100	100	100
MRS	+	0	15	100	100	100	100	100
NB	-	0	7	45	55	65	80	100
NB	+	0	6	85	90	93	98	99
BHI	+	0	100	100	100	100	100	100
NZA	+	0	100	100	100	100	100	100

#: Mean of duplicate analyses from one of the two experiments.
O: Oxygen, -, static; +, shaking

NZA: N-Z amine medium; MRS: Mann-Rogosa-Sharpe medium;
NB: Nutrient broth medium; BHI: Brain Heart Infusion medium

(Tables 6.12, 6.17). The drop in pH in samples inoculated with both organisms was similar to that observed in samples inoculated with just one of the organisms (Tables 6.4,6.5,6.7, 6.15,6.16). Moreover the pH drift was accentuated when glucose was added to MRS and NB (Table 6.15, 6.16) with either static and shaking cultures.

Products of metabolism

Exoprotein

In MRS or NB without additional glucose, the proteins in the medium decreased throughout incubation (Table 6.15) in both shaking and static cultures. In contrast when a medium was supplemented with glucose, there was an increase in the concentration of exoproteins. This increase was more pronounced in MRS than in NB. It was observed that the rate of exoprotein production was higher in MRS than in NB, while the rate of exoprotein decrease in MRS without glucose was slower than in NB (Table 6.15). This glucose effect on exoprotein production was not found with the other media (NZA, BHI)- (Table 6.16).

Lactic and acetic acid

In general, the results (Tables 6.15,6.16) obtained with lactate production were similar to those reported earlier for samples inoculated with a single organism (Tables 6.4, 6.5, 6.6, 6.7). It needs to be stressed, however, that the production of acetic acid in samples inoculated with both organisms was almost twice that in samples inoculated with single organisms (Table 6.13) under static conditions.

Effect of aeration

Although the presence of oxygen (shaking samples) increased the rate of lactate utilization (Tables 6.15, 6.16), the exoprotein profile was not influenced significantly (Figs 6.1,6.2,6.3 and 6.4)

Discussion

Of the results presented above, those relating to Lowry-positive materials and exoproteins as measured by the Coomassie blue method were inconsistent. I am of the opinion that neither is of importance when considered in the context of sausage production when either *L. plantarum* or *St. carnosus* are used as starter cultures.

It is well known that with modified atmospheres lactic acid bacteria limit the shelf life of packaged meat and meat products during refrigerated storage by the formation of off-flavours (Egan 1983). On the other hand, lactic acid bacteria are used as starter cultures in the processing of fermented meat products in order to obtain a desirable aroma, flavour and texture as well as preservation (Lucke and Heckelmann 1987; Nychas and Arkoudelos 1990a). *Staphylococcus carnosus* would also be expected to affect the quality of the final product.

Until recently there was a concensus of opinion that lactic acid bacteria were either homo- or heterofermentative in the sense that the former produced *ca.* 2 lactic acid molecules from 1 glucose molecule and the latter produced 1 lactic acid, 1 acetic acid (ethanol) and 1 CO_2. It is now recognised, however, that environmental changes may cause the fermentation of the former may switch to that of the latter (Sedewitz *et al.* 1984; Borch *et al.*1991; Marshall 1992). Thus the metabolism of lactic acid bacteria and that of staphylococci also in meat products may well be affected by environmental factors, thereby influencing their beneficial/detrimental contribution to such products. This switch is now well recognised by dairy microbiologists (Marshall 1992).

The results presented above indicate that the change from homo- to heterolactic fermentation occurred in my experiments. Pyruvate, the 'key' intermediate metabolic

product, can be channelled to compounds which impart aroma and/or flavour. Moreover acetic acid has a different flavour and a greater antibacterial action than that of lactic acid. The type of energy source (glucose or galactose), glucose limitation, the degree of aeration, the concentration of lactate dehydrogenase (LDH), NADH peroxidase or fructose 1,6-diphosphate can influence the conversion of lactate to acetate (Garvie 1980; Kandler 1983; Sedewitz *et al.* 1984; Thomas *et al.*1979; Murphy and Condon 1984a,b; Borch and Molin 1989; Cogan *et al.* 1989; Tseng and Montville 1990). In addition to these factors, amino acids and lactate are reported to affect also the type and the patterns of fermentation products (Thomas *et al.* 1979; Borch & Molin 1989; Nychas *et al* 1991b; Nychas and Board 1991). For example both glucose or oxygen limitation may cause the switch noted above (Thomas *et al.* 1979; Sedewitz *et al.* 1984; Murphy *et al.* 1985) in certain lactic acid bacteria and influence the exoprotein profile also (Nychas *et al.* 1991b) in staphylococci.

It is well known that lactate is the main end product of lactic acid bacteria not subjected to environmental stress. This product, however, could be further metabolised to acetate under aerobic conditions by stereospecific NAD-independent, flavin-containing lactate dehydrogenase or lactate oxidase (Kandler 1983). Indeed among 23 lactobacilli tested by this worker, 21 strains were found to oxidise lactate. These oxidisers could be divided into two groups. One possesed flavin-containing L-lactate oxidase which used oxygen as an electron acceptor thereby producing hydrogen peroxide as well as acetic acid. In the laboratory the second required methylene blue as an electron acceptor in order to perform lactate oxidation at significant levels. It is for these reasons that some species of *Lactobacillus* are described as facultatively heterofermentative.

Sedewitz *et al.* (1984) found that *Lactobacillus plantarum* produced acetic in addition to lactic acid under aerobic conditions. The latter was produced initially and, when the carbohydrate content of the medium was approaching exhaustion, lactic acid was metabolised further to acetic acid. The accumulation of this acid could proceed from further metabolism of L-lactate by the iLDH enzyme. This enzyme exists in many bacteria (Garvie 1980) and converts L-lactate to pyruvate. As yet no evidence of a reverse reaction has been reported. Although the NAD-linked, lactate dehydrogenase (iLDH) (Garvie 1980) is able to function under both aerobic and anaerobic conditions (Collins and Lascelles 1962; Garrard and Lascelles 1968; Morse and Baldwin 1971; Blumental 1972; Gotz and Schliefer 1975; 1976; Sedewitz *et al.*1984), acetic acid production by lactobacilli is limited. Another enzyme involved in further metabolism of pyruvate to acetic under aerobic metabolism is pyruvate oxidase (Sedewitz *et al.*1984; Kandler 1983; Murphy *et al.* 1985).

Staphylococci also could produce acetic acid at the expense of lactic acid. This is in accord with the observations of Nychas *et al.* (1991b) with *St. aureus*. Indeed as the lactate dehydrogenase can function under both aerobic and anaerobic conditions, and as such may be able to channel carbon skeletons from the TCA cycle through to lactate. In general, in the absence of a readily available source of energy, *St. carnosus* and *L. plantarum* cultures grown in various media appeared from this study to make use of iLDH by using lactate as a carbon source oxidising it further to acetic acid (lactobacilli, staphylococci) or to stimulate the TCA cycle (staphylococci- Blumental 1972) which may account for the decrease in lactate observed not only in BHI medium but in the other media also. Indeed, in this part of the present study it was observed that lactate, although it was produced initially in the various media

under static or shaking conditions, was utilised in the later stages of incubation in all samples inoculated with these two organisms in combination or alone. The presence of glucose in static cultures (Table 6.13 and Figs 6.5, 6.6) as well as oxygen limitation induced this heterolactic switch. This is in accordance with the findings of Borch and Molin (1989).

The findings in this part of the study are very important to our understanding of the physiology of these two organisms when they are used as starter cultures. The possibility of acetic acid instead of lactic acid in fermented sausages could be beneficial particularly from the public health point of view.

General Discussion

The studies of the microbiology and physicochemical attributes of meats 'beef or pork' obtained from Greek butchers provided information that is not readily interpreted in the light of current knowledge about meat microbiology in general (Nottingham 1982; Gill and Mollin 1991). The high pH and low glucose and lactic acid concentrations of some of the meat might well have lead me to the conclusion that it ought to considered to be DFD meat (Gill 1982, 1986). In practice such a definition seems improbable in view of the gluconate content of some of the meat used in this study. Thus low concentrations of glucose were associated with high gluconate concentrations and also high pH. As gluconate is an oxidation product of glucose by pseudomanads, it is tempting to speculate that the low glucose content of the high pH meat is not a consequence of an animal being stressed immediately prior to slaughter - a common cause of DFD - but due to the activities of pseudomonads.

It is tempting to speculate that some features of the Greek methods of handling meat at the retail outlet may be involved. It was noted previously that meat in Greece is commonly held at ambient whilst the butcher's shop is open. Could it be that a rapid rise in the surface temperature of meat taken from a chill together with rapid glucose diffusion from within a carcass causes extensive glucose - gluconate conversion by pseudomanads? These organisms were dominant on high pH meat at the time of purchase. This contention would not account for all the observations. The activities of pseudomonads would not be expected to influence lactic acid concentration during the period when glucose and gluconate were plentiful (Gill 1976, 1982). Thus future work needs to be done to elucidate this paradox. In general the microbiological and physicochemical changes occurring in Greek meat packed in modified atmospheres were analogous to those reported elsewhere (Daniels *et al.* 1985). In

other words, the unusual feature noted above did not cause unusual changes during the MA storage of minced meat.

The survey of fermented Greek sausages of the salami type (Chapter 4) revealed the widespread occurrence of *Staphylococcus carnosus* in sausages but not in the factory environment. As Greek law forbate the use of starter cultures at the time that this study was done, it was assumed that the traditional back-slop tecnhique practiced in Greece favoured the selection of this organism. Presumably traditional methods have favoured the growth of this organism in sausages made in Germany also (Bacus 1984, 1986; Lucke 1985a, b). A recent study in Thailand (Tanasupawat *et al.* 1991) showed that *St. carnosus* is a common member of the flora developing in a range of fermented fish containing >5% or soy sauce made with >17% NaCl. Thus it can be concluded that this organism is not peculiar to fermented sausages but probably has a worldwide distribution in fermented products rich in proteins. As yet the factors favouring the growth of this organism in such products have not been identified.

Our forefathers established that the addition of fermentable carbohydrates to diced or minced meat led to a fermentation and hence preservation (Lucke 1985a, b). As noted above *St. carnosus* is now beginning to be recognised as an important fermentative organism in this process. In the present investigation various methods were tried to study sausage fermentation in the laboratory. Only one method proved satisfactory, sausage meat stuffed in natural casings and stored in an incubator with moving air during fermentation. Sausage fermentation by this method was aided by inoculation of the meat with both *L. plantarum* and *St. carnosus*. It would appear therefore that the lactobacilli present on Greek meat obtained from butchers (Chapter 3) were poorly adapted to the fermentative environment found in sausage meat. Studies with a variety of laboratory media established that *L. plantarum* and *St. carnosus* in

combination brought about a more rapid glucose fermentation than either organism alone. Thus it was concluded that manufacturers ought to use starter cultures of both organisms in sausage production. This study of meat fermentation in the laboratory as well as the behaviour of the *Lactobacillus* and *Staphylococcus* in laboratory media highlighted a feature that has only recently been mentioned in the literature on fermented meat or milk (cheese) products (Marshaall 1992; Tseng and Montville 1990; Borch and Agerhem 1992; Borch *et al.* 1991), namely a switch from homo- to heterolactic fermentation by a homolactic organism such as *L. plantarum*. Glucose depletion appears to be the important cause of this switch. It is well known that this switch is a common feature in cheese production (Marshall 1992; Olson 1990; Crow and Turner 1986; Turner *et al.* 1983). As it is evidently common in meat products also, future studies ought to be concerned with the effect of these products of an amended fermentation, particularly acetic acid, on both flavour and self life of sausages.

APPENDIX I

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Microbiological and physicochemical changes in minced meats under carbon dioxide, nitrogen or air at 3°C

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Summary

Microbiological changes were studied in minced beef and minced dark, firm, dry (DFD) pork stored under different atmospheres (100% in carbon dioxide, nitrogen or air) at 3°C. The storage life (time for 100-fold increase in TVC) of samples flushed with carbon dioxide was increased by *c.* 3–4 days. Pseudomonads were the dominant organisms in samples stored in air, and lactic acid bacteria and *Brochothrix thermosphacta* in those stored under carbon dioxide. There was an alkaline drift in all samples, but at different rates (air > nitrogen > carbon dioxide). The rate of glucose assimilation in normal and DFD beef was slower in the samples stored under carbon dioxide than those under nitrogen or air. Lactate, gluconate, acetic acid, ethanol and diacetyl occurred in normal and DFD beef regardless of the storage atmospheres.

Keywords

Beef, DFD beef, metabolite changes, modified atmosphere packing, pork.

Introduction

Fresh beef or pork and their products are packed in modified atmospheres (MA) to sustain visual appearance and to extend shelf-life (Erichsen & Molin, 1981; Blickstad & Molin, 1983). In northern EC countries such as Denmark and UK, MA have been used for over 25 years, but the technique is still in its infancy elsewhere. The tradition that Greek consumers buy their meat from local butchers and the absence of large supermarket chains are the principal reasons for the limited exploitation of MA systems in Greece. Carbon dioxide, nitrogen, oxygen and, for experimental purposes, carbon monoxide have been used in modified atmosphere packs (Clark *et al.*, 1976). Nitrogen is biologically inert at normal atmospheric pressure, whilst carbon dioxide affects the growth and composition of the contaminating flora and the germination of bacterial spores (Enfors & Molin, 1978; Blickstad & Molin, 1983; Daniels *et al.*, 1985; Jones, 1989). It has been found that at the end of storage life *Brochothrix thermosphacta* (*Microbacterium thermosphactum*) and lactic acid bacteria were the major contaminants on beef, pork or lamb stored in modified atmospheres of 20–100% of CO_2

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(Newton *et al.*, 1977; Erichsen & Molin, 1981; Nychas & Board, 1985). On air-stored samples, where oxygen is present, *Pseudomonas* spp. dominated (Enfors *et al.*, 1979; Nychas & Board, 1985). Nitrogen alone was found to have no effect on bacterial growth on meat (Enfors *et al.*, 1979). While the microbiological changes, with the exception of yeasts, are well established, the chemical changes accompanying the growth of bacteria on meat during storage are poorly understood.It was suggested that the metabolism of glucose, lactic acid, certain amino acids, nucleotides, urea and sarcoplasmic proteins can all occur during storage (Gill, 1976, 1986; Jay, 1986). The off-odours formed on spoilage of meat have been attributed to volatile short-chain fatty acids produced by bacteria from these substrates (Dainty & Hibbard, 1983; Dainty *et al.*, 1985). The identity of the end products of bacterial growth on meat or poultry stored under vacuum or in air has been well established (Jay, 1986), but there is little information about the products produced during carbon dioxide and nitrogen storage. There is pressure on the small food manufacturers in several countries to adopt the EC specifications for quality and safety standards. In anticipation of the adoption of 'new' technologies, the objectives of the present study were to measure the extension of microbial shelf-life produced by MA storage of mince from normal and high pH (dark, firm, dry; DFD) Greek beef and pork, and to confirm previous work relating spoilage to glucose and gluconate metabolism, and to changes in other muscle and microbial metabolite end products.

Materials and methods

Minced beef of normal pH and DFD (pH > 6.2) pork and beef were used; for each approximately 2×5kg of minced meat was purchased around 09.00 h. It was transported to the laboratory within 30 min of purchase and held at about 1°C for 1–2h until it was portioned (*c.* 200–250g) into 48 rigid plastic (Dyno Norway, cat. pac 515 3) containers. Each container was enclosed in a polyethylene bag [oxygen permeability, $P=2.88 \times 10^{-10}$ cm^3 (STP) cm cm^{-2} s^{-1} cm Hg^{-1}] which was evacuated and flushed three times before filling with 100% CO_2, N_2 or air, and double heat sealing leaving a gas space of approximately 4 litres. The 16 containers used with each atmosphere were stored at 3±0.2°C for *c.* 2 weeks. On every sampling day two plastic containers from each treatment were removed for analysis.

Microbiological analysis

Samples (25g) from mixed bulk minced meat were weighed out aseptically, sterile 1/4 strength Ringer's solution (225ml) added and mixed with a stomacher (Lab Blender 400, Seward Medical, London, UK) for 60s at room temperature. Decimal dilutions in 1/4 strength Ringer's solution were prepared and duplicate 1 ml or 0.1 ml samples of appropriate dilutions mixed or spread on the following agar media for the isolation of particular groups of bacteria or yeasts: (a) Total viable count (TVC) on plate count agar (PCA; Oxoid), incubated at 20°C for 96h; (b) Enterobacteriaceae and coliforms on VRBG (Oxoid) and VRBA (Oxoid) respectively, with an overlay, incubated at 30°C for 24h; (c) *Brochothrix thermosphacta* on Gardner's (1966) STAA medium made from basic ingredients in the laboratory, incubated at 20°C for 96h; (d) Yeasts, on Rose Bengal Chloramphenicol agar medium (RBC; Oxoid), incubated at 25°C for 96h; (e) Lactic acid bacteria on MRS (Oxoid), overlayed with the same medium and incubated at 25°C for 96h, under anaerobic conditions; (f) *Pseudomonas* spp. on Mead & Adams (1977) CFC medium (Oxoid), incubated at 15°C for 48h.

Physicochemical analysis

Immediately after microbiological sampling, the pH of the diluted mixed mince sample was measured and the extract release volume (ERV) was determined as described by Jay (1964).

Chemical tests. Minced meat (25 g) was reduced to a fine suspension with 100 ml cold water (3–5°C) in an Omni mixer (Waring, New Hartford, UK). The suspension was agitated (orbital shaker, 100 r.p.m., 45 min) at 3°C, centrifuged (4000 × *g*; 5 min; 3°C), filtered and the clear filtrate used to determine total sugars (Roe, 1954), glucose (GOD-perid method, Boehringer, Mannheim,FRG), ammonia (Chavey & Marbach, 1962) and soluble proteins (Lowry *et al.*, 1951). Volatile fatty acids (VFA) and other volatile compounds in the filtrate were determined by gas chromatography as follows. Clear filtrate (10 ml) was filtered through a bacteriological filter (0.2 μm) and stored at −80°C until analysed. The thawed sample (10 μl) was injected directly into a 2 m × 6.25 mm column (10% SP-1000, 1% H_3PO_4 100–120 Mesh Chromosorb W-AW and programmed from 90°C to 180°C at 4°C min^{-1} with He (40 ml min^{-1}) as carrier gas and a Flame Ionization Detector (FID). Peak identification was by comparison of retention times with authentic compounds on the above and on Chromosorb 101 80–100 Mesh.

Lactate and gluconate were analysed in the minced meat by the method of Gutmann & Wahlefeld (1974) and Mollering & Bergmeyer (1974) respectively. To deproteinize samples, minced meat was added to 5 ml of ice-cold 1 N perchloric acid, shaken vigorously and centrifuged (15 min, 4000 × *g*).

Results

Microbiological changes

The initial microflora of normal beef and DFD pork (Table 1) comprised, in decreasing order of magnitude, *Pseudomonas* spp. *Br.thermosphacta*, lactic acid bacteria, yeasts and Enterobacteriaceae. Similar microbiological results were obtained from DFD minced beef (results not shown). It is evident that the type of meat species (beef or pork) and/or type of meat (normal, DFD) did not affect the composition of the initial microflora. Storage in carbon dioxide (100%) instead of air reduced the increase in TVC and extended the shelf-life, taken as the time (days) required for an increase of 2 log units. For beef and DFD pork, it was 6–8 days more than that in air or in nitrogen. In general, it was found that Gram-negative bacteria (*Pseudomonas* spp., Enterobacteriaceae and coliforms) and particularly yeasts on DFD pork were the most CO_2 sensitive members of the initial microbial species on these samples (Table 1), whereas the populations of Gram-positive bacteria, particularly *Br. thermosphacta*, were equal to or greater than those of pseudomonads on meat samples stored in CO_2. Under nitrogen, only Enterobacteriaceae were inhibited, and, after 9 days, the numbers of Gram-positive bacteria and yeasts were equal to, or higher than, those in samples stored in air. With samples stored in air, *Pseudomonas* spp. became dominant on both meats.

Physicochemical changes in normal and DFD beef

By the eleventh day, minced beef in air was well spoiled, and bacterial counts were determined to confirm any trends compared with those stored in CO_2 and N_2.

On the first day, analyses showed that the concentations of glucose, total sugars, lactate and soluble proteins were higher in normal than in DFD beef. A rapid loss of glucose and total sugars occurred later in normal beef under carbon dioxide than in

Table 1. Changes in microbial population (c.f.u. g^{-1}) in Greek minced beef and DFD pork on storage in air, carbon dioxide or nitrogen at 3°C

Modified atmosphere packing	Air				CO_2				N_2			
	Day of sampling				Day of sampling				Day of Sampling			
	1	3	5	9	3	5	9	11	3	5	9	11
Normal minced beef												
Total viable count	6.6	7.5	8.6	9.9	7.3	6.8	7.6	8.4	8.5	8.5	9.9	10.5
Pseudomonads	5.4	6.9	8.1	9.6	5.7	6.4	7.5	7.4	7.2	8.2	9.6	10.2
Brochothrix thermosphacta	4.5	5.9	7.9	8.9	5.4	6.0	8.5	8.7	6.9	7.7	8.9	10.0
Lactic acid bacteria	3.5	3.8	4.8	6.2	3.9	4.8	6.5	7.2	4.4	5.4	6.7	7.6
Yeasts	3.4	3.9	4.9	6.5	3.4	3.9	4.8	5.7	4.9	4.7	6.2	7.3
Enterobacteriaceae	2.9	4.4	6.2	7.7	3.5	4.2	4.7	5.0	5.5	5.4	6.9	7.5
Coliforms	3.0	2.6	4.3	5.8	2.3	2.7	3.0	2.8	3.2	3.5	4.8	5.5
DFD minced pork												
Total viable count	6.7	8.5	9.4	9.8	7.5	8.2	8.0	8.6	8.2	8.8	9.3	9.8
Pseudomonads	6.5	8.4	9.0	9.4	7.0	7.6	7.8	8.4	8.1	8.7	8.9	9.5
Brochothrix thermosphacta	6.0	7.7	8.2	8.7	6.6	7.5	7.8	8.5	7.2	9.1	9.0	9.2
Lactic acid bacteria	5.4	7.1	7.3	7.7	5.7	6.0	6.4	7.4	6.9	7.4	7.8	8.0
Yeasts	4.8	5.6	6.7	7.0	4.7	4.7	4.8	5.2	5.3	6.6	7.0	7.3
Enterobacteriaceae	2.8	4.2	6.1	6.8	3.7	3.7	4.2	4.9	4.7	5.8	6.5	6.7

nitrogen or air (Table 2). This was not demonstrated in DFD beef, which contained much less glucose (Table 3). Changes in the much lower (c. 1000-fold) levels of gluconate were small in normal minced beef, but the higher initial levels in DFD minced beef were lost rapidly in air, more slowly in N_2 and were delayed in CO_2. Increase in pH occurred rapidly in both types of beef in air, more slowly in nitrogen and hardly at all in carbon dioxide (Tables 2 and 3). The increases were paralleled by a decrease in lactic acid in normal, but not in DFD beef. Ammonia and soluble proteins tended to increase, and ERV to decrease in all samples. After 9 days of storage in CO_2, the amount of lactic acid was always greater than in nitrogen or air.

Volatile compounds

Ethanol, diacetyl, acetic acid and an as yet unidentified compound (Table 4) were always present in minced meat sampled throughout storage under different gaseous environments but there was no evidence that they were related to off-odours. In addition acetoin, propionic acid, *iso*-butyric, *iso*-valeric, butyric, valeric, *iso*-caproic, and caproic acids were present in minced beef with off-odours. The unknown compound was the dominant peak at the end of storage of both beef samples in carbon dioxide and in normal beef stored in nitrogen.

Discussion

When Greek minced beef or DFD pork was stored in CO_2, the shelf-life at 3°C, defined as 100-fold increase in TVC, was almost doubled; the growth of Gram-negative bacteria and yeasts was inhibited and *Br. thermosphacta* and lactic acid bacteria became dominant. These results agree with those previously reported for bacteria on beef, lamb

Table 2. Physicochemical changes* during storage of naturally contaminated ground beef with normal pH stored at 3°C under different gaseous atmospheres

Gas mix used	Storage days	pH	Extract release volume (ml)	Glucose (mg 100g^{-1})	Total sugars (mg 100g^{-1})	Lactate (mg 100g^{-1})	Gluconate (μg g^{-1})	Soluble proteins (mg 100g^{-1})	Ammonia (A625)
	1	5.5±0.051**	82±2.1	111±5.3	150±6.2	380±7.1	19±0.8	107±0.80	0.08±0.001
Air	3	5.5±0.052	78±1.4	97±5.2	120±3.4	304±8.3	23±0.5	87±0.75	0.11±0.002
	5	5.6±0.091	77±2.6	91±6.1	110±2.6	241±5.0	22±0.4	123±0.50	0.13±0.002
	9	6.4±0.078	50±3.7	29±2.5	70±5.8	117±4.5	16±0.2	154±1.0	0.25±0.003
	11	6.2±0.050	15±3.2	13±3.0	50±8.1	87±6.5	12±0.8	112±0.55	0.17±0.002
CO_2	3	5.5±0.034	79±3.3	91±4.2	140±7.2	361±6.2	20±0.8	140±1.2	0.08±0.005
	5	5.5±0.050	76±4.5	91±5.3	145±3.7	300±5.5	12±0.2	145±0.80	0.11±0.001
	9	5.7±0.052	55±2.7	81±3.6	150±5.5	252±1.3	25±0.4	123±1.4	0.11±0.004
	11	5.7±0.040	85±5.2	61±4.3	160±5.3	317±4.5	16±0.5	35±0.90	0.13±0.002
N_2	3	5.6±0.054	60±2.1	58±5.0	130±6.3	375±2.3	53±0.9	143±0.9	0.10±0.004
	5	5.6±0.035	60±4.4	30±4.4	100±5.9	305±1.5	21±0.5	154±1.2	0.12±0.002
	9	6.5±0.065	42±4.1	22±3.1	100±7.2	197±2.9	14±0.4	156±1.7	0.14±0.004
	11	6.1±0.045	45±5.3	36±6.0	40±4.1	201±1.5	12±0.3	132±0.70	0.16±0.003

*Mean (±s.d.) of two samples from the same treatment (two determinations for each sample).

Table 3. Physicochemical changes during storage of naturally contaminated minced beef with DFD characteristics stored at 3°C under different gaseous atmospheres

Gas mix used	Storage days	pH	Extract release volume (ml)	Glucose (mg $100g^{-1}$)	Total sugars (mg $100g^{-1}$)	Lactate (mg $100g^{-1}$)	Gluconate (μg g^{-1})	Soluble proteins (mg $100g^{-1}$)	Ammonia (A625)
	1	6.25±0.051*	64±4.2	65±6.1	110±3.1	160±4.7	94±0.9	5±0.3	0.10±0.002
Air	3	6.6±0.083	35±4.8	34±3.3	80±7.3	132±4.5	11±0.4	10±0.7	0.10±0.003
	5	7.2±0.075	22±5.0	26±4.1	80±3.4	110±6.0	4±0.6	65±1.2	0.12±0.006
	9	6.9±0.055	22±2.2	15±1.9	72±3.2	84±3.4	7±0.2	98±1.2	0.14±0.002
	11	6.8±0.015	14±3.3	10±2.2	42±2.1	71±4.5	4±0.5	154±1.4	0.17±0.003
CO_2	3	6.3±0.052	42±2.1	57±3.1	90±2.3	170±9.1	95±0.8	6±0.3	0.10±0.004
	5	6.5±0.054	64±3.9	44±4.1	95±5.3	132±4.3	85±0.3	3±0.1	0.12±0.003
	9	6.4±0.051	43±2.3	30±3.3	85±3.5	136±5.4	132±0.7	6±0.5	0.12±0.006
	11	6.6±0.053	20±3.4	17±1.5	60±4.3	100±3.9	30±0.5	63±1.4	0.14±0.005
N_2	3	6.5±0.054	34±4.5	35±2.4	100±6.2	144±8.0	70±0.7	8±0.3	0.10±0.007
	5	6.5±0.045	66±6.3	31±2.2	75±5.4	152±4.4	41±0.6	87±1.1	0.13±0.003
	9	7.1±0.035	29±3.4	35±3.3	60±4.3	78±7.2	6±0.9	126±1.3	0.16±0.005
	11	6.9±0.055	20±2.8	20±3.1	35±5.3	92±2.2	5±0.3	187±0.9	0.20±0.005

*Mean (±s.d.) of two samples from the same treatment (two determinations for each sample).

Table 4. Volatile compounds* produced by the naturally contaminated ground beef with normal pH 5.5 and high pH 6.25 under different conditions

Gas mix used	Storage days	Ethanol	Diacetyl	Unknown	Acetic acid
Normal pH 5.5					
	1	28.0±1.5	0.1±0.01	10.6±0.1	61.0±2.0
Air	3	16.0±2.1	0.9±0.05	52.2±0.3	30.0±2.1
	5	2.3±0.2	0.5±0.00	81.2±1.0	16.0±1.6
	9	2.3±0.2	0.1±0.00	65.5±1.2	32.0±1.9
	11	49.7±0.9	7.2±0.02	15.2±0.6	27.0±1.3
CO_2	3	20.3±1.2	0.8±0.01	36.8±1.1	42.0±2.3
	5	1.9±0.2	3.8±0.02	80.0±1.9	14.0±2.6
	9	1.2±0.1	0.3±0.01	96.4±2.3	2.1±0.1
	11	2.0±0.2	0.1±0.01	92.5±2.5	5.2±0.5
N_2	3	6.7±0.4	0.5±0.01	50.2±1.5	42.0±3.2
	5	2.0±0.1	0.2±0.00	89.6±3.1	8.0±0.3
	9	1.5±0.2	0.1±0.01	86.0±2.2	12.6±0.2
	11	1.9±0.2	0.2±0.00	91.7±2.3	6.2±1.1
High pH 6.25					
	1	7.0±0.3	1.0±0.01	29.0±0.9	63.0±0.9
Air	3	5.9±0.3	0.7±0.00	45.0±2.1	48.0±2.3
	5	6.5±0.2	7.4±0.20	23.0±1.5	63.0±3.4
	9	9.5±0.3	2.7±0.10	40.3±2.3	47.5±2.7
	11	11.7±0.4	1.1±0.10	42.3±1.9	45.2±1.6
CO_2	3	12.3±0.6	0.9±0.00	30.5±2.0	56.3±2.2
	5	6.8±0.4	2.5±0.02	57.5±1.5	33.3±3.4
	9	3.7±0.5	0.4±0.00	86.0±2.5	10.0±0.7
	11	5.2±0.3	0.3±0.01	91.0±3.1	3.0±0.3
N_2	3	9.1±0.4	0.7±0.01	64.3±2.3	25.4±0.3
	5	5.5±0.3	0.6±0.05	69.0±2.5	25.0±0.5
	9	12.0±0.4	2.1±0.10	54.0±3.4	32.0±0.9
	11	1.9±0.1	0.2±0.02	64.0±1.7	32.0±0.6

*Values are the mean (±s.d.) of three replicates from each sample. These are expressed as percentage of the total which derived from the sum of their areas. These areas are given by the integrator (Perkin-Elmer 10B) which was linked with the 3B Perkin Elmer gas chromatograph.

and pork (Newton *et al.*, 1977; Erichsen & Molin, 1981; Blickstad & Molin, 1983) and add new data about the growth inhibition of meat yeasts by CO_2. Nitrogen did not retard total microbial growth on beef or DFD pork, as shown by Enfors *et al.* (1979).

As carbon dioxide selects a microbial flora different from that of minced meat stored in air or nitrogen, some differences in physicochemical properties might be expected. Jay (1964) and Shelef & Jay (1970) introduced Titrimetric Acidity and ERV, while Sutherland *et al.* (1975) also used pH for the prediction of bacterial spoilage of fresh beef. Our results in normal beef stored in air (Tables 1, 2 and 3) agree with those of Jay (1964), in that changes in ERV approximately decreased with microbial growth; in contrast, those in carbon dioxide did not. Carbon dioxide, and to a lesser extent nitrogen, influenced pH without appreciable changes in ERV; similar results have been

reported for vacuum-packaged beef (Sutherland *et al.*, 1975). The failure of these tests as indicators of spoilage in minced meat in carbon dioxide is due both to its effects on the composition of the flora, and as a weak acid (Jones, 1989), on the pH of the meat.

The role of glucose in meat microbiology is well established (Gill, 1976; Nychas *et al.*, 1988). The conversion of glucose to gluconate via the extracellular glucose–dehydrogenase of pseudomonads provides them with a competitive advantage because gluconate is not so readily utilized by other members of the meat microflora (Farber & Idziak, 1982; Nychas *et al.*, 1988). The delay in the utilizition of glucose and total sugars, as well as the decrease of gluconate during the early stages of storage of both types of minced meat stored in carbon dioxide, may have been due to high P_{CO_2} or low P_{O_2} inhibiting the activity of glucose–dehydrogenase of pseudomonads (Mitchell & Dawes, 1982; Nychas *et al.*, 1988). The subsequent increase in the concentration of gluconate (Tables 2 and 3) was associated with an increase in the size of the population of pseudomonads (Table 1). It would appear therefore that, during storage, the composition of the gas phase in the bags changed, possibly due to the diffusion of O_2. These changes in gluconate concentration contrast with those occurring in meat stored in either a normal atmosphere or nitrogen. In both instances the concentration of glucose and total sugars diminished rapidly, and the transient peak in gluconate concentration occurred at an early stage of storage (Tables 2 and 3).

Gluconate is not connected directly with the production of off-odours, which are mainly caused by sulphur compounds, diacetyl, lactic acid, acetic acid and to a lesser extent to short-chain branched fatty acids (e.g. *iso*-butyric, *iso*-valeric) and to alcohols (e.g. 2,3 butanediol, 2-methylpropanol) (Dainty & Hoffman, 1983; Dainty *et al.*, 1985). The putrid off-odour, which was evident in minced meat stored in air, coincided with the increase of ammonia and soluble proteins and glucose diminution (Table 2; ninth day in both experiments), and is in accord with the findings of Gill (1976) and Gill & Newton (1977). As CO_2 inhibited growth as well as glucose assimilation by pseudomonads, the dairy/cheesy odours mainly found in samples stored in CO_2 and, to a lesser extent, in N_2 might be produced by *Br. thermosphacta* which can produce diacetyl and alcohols (Dainty & Hibbard, 1983; Dainty & Hoffman, 1983) exclusively from glucose under aerobic conditions or under low O_2 tension (Blickstad & Molin, 1984). Therefore spoilage of stored minced meat is not, as might be inferred from the findings of Gill (1976, 1986) and Gill & Newton (1977), exclusively associated with the onset of amino acid metabolism subsequent to glucose and gluconate depletion by pseudomonads.

Although the presence of off-odours is the most rapid and universally accepted indicator of the end of shelf-life of meat stored in air, problems may arise with novel forms of gas packaging. This study has shown that the important changes in microbial metabolites, and their possible role as indicators of incipient spoilage, are determined by storage methods. Indeed, this study demonstrated clearly that each different type of storage condition (air, CO_2, nitrogen) provided unique environments for the growth of particular groups of bacteria.

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